

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

NEURONAL APOPTOSIS INHIBITOR PROTEIN, GENE SEQUENCE
AND MUTATIONS CAUSATIVE OF SPINAL MUSCULAR ATROPHY

FIELD OF THE INVENTION

5 The gene for the neuronal apoptosis inhibitor protein (NAIP) has been identified in the q13 region of chromosome 5. Mutations in this gene have been diagnosed in individuals with Type I, II and III Spinal Muscular Atrophy. The amino acid sequence of the neuronal
10 apoptosis inhibitor protein is provided and homology to viral apoptosis proteins demonstrated.

BACKGROUND OF THE INVENTION

15 In order to facilitate reference to various journal articles in the discussion of various aspects of this invention, a complete listing of the reference is provided at the end of the disclosure. Otherwise the references are identified in the disclosure by first author's name and publication year of the reference.

20 The childhood spinal muscular atrophies (SMAs) are a group of autosomal recessive, neurodegenerative disorders classified into three types based upon the age of onset and clinical progression (Dubowitz et al., 1978; Dubowitz et al., 1991). All three types are characterized by the degeneration of the alpha motor neurons of the spinal
25 cord manifesting as weakness and wasting of the proximal voluntary muscles. Type I SMA is the most severe form with onset either in utero or within the first few months of life. Affected children are unable to sit unsupported and are prone to recurrent chest infections due to
30 respiratory insufficiency, thus rarely surviving the first few years of life (Dubowitz et al., 1978; Dubowitz et al., 1991). This acute form, with a carrier frequency of 1/60 to 1/80, is one of the most frequent fatal autosomal recessive disorders. Affected children with
35 Type II SMA never walk unaided and although the prognosis is variable, such children may die in adolescence. Those affected with Type III SMA maintain independent ambulation but develop weakness any time between the age of 3 to 17 years manifesting a mildly progressive course

(Dubowitz et al., 1978; Dubowitz et al., 1991).

In 1990, all three childhood forms of SMA were genetically mapped to the long arm of chromosome 5 at 5q11.2 - 13.3 (Brustowicz et al., 1990; Gilliam et al., 1990; Melki et al., 1990). Subsequent multi-point linkage analyses and the identification of recombinant events have further localized the genetic defect to the region flanked centromerically by D5S435/D5S629 (Soares et al., 1993; Wirth et al., 1993, Clermont et al., 1994)) and telomerically by MAP1B/D5S112 (Wirth et al., 1994; MacKenzie et al., 1993; Lien et al., 1991). This interval has been refined by the more recent identification of recombination events indicating that the SMA gene lies distal to CMS-1 (Yaraghi et al., submitted to *Human Genetics*; van der Steege, et al., submitted to *Human Genetics*) and proximal to D5S557 (Francis et al., 1993). We and others have detected chromosome 5-specific repetitive sequences with particular abundance in the D5S629/CMS-D5S557 region (Francis et al., 1993; Thompson et al., 1993) which has impeded the isolation and ordering of both clones and simple tandem repeats. An array of cosmid clones spanning the 200 kb CMS-1 (Kleyn et al., 1993)/CATT-1 (Burghes et al., 1994, McLean et al., in press)/D5F150/D5F149/D5F153 (Melki et al., 1994) region within this interval has been constructed.

We established a contiguous array of YAC clones encompassing the SMA containing D5S435 - D5S112 interval of 5q13.1. We then discovered a gene within this interval of 5q13.1 which coded for a neuronal apoptosis inhibitor protein (NAIP). Further studies demonstrated that a deletion in this gene was found in Type I, II and III Spinal Muscular Atrophy.

SUMMARY OF THE INVENTION

A gene encoding a neuronal apoptosis inhibitor protein (NAIP) was discovered in the q13 region of human chromosome. According to an aspect of the invention, the

cdna sequence coding of the neuronal apoptosis inhibitor protein is provided and set out in Table 4. According to another aspect of the invention, the predicted amino acid sequence of the neuronal apoptosis inhibitor protein is provided from the cdna sequence.

According to another aspect of the invention, a deletion of the neuronal apoptosis inhibitor protein gene was discovered in persons with Type I, II and III Spinal Muscular Atrophy disease. The discovery of the neuronal apoptosis inhibitor protein gene deletion provides a diagnostic indicator for use in the diagnosis of Spinal Muscular Atrophy.

In order to facilitate a further description of various aspects of the invention, reference will be made to various Figures of the drawings. A brief description of the drawings follows this invention summary section.

According to a further aspect of the invention, a human gene is provided which maps to the SMA containing region of chromosome 5q13. The gene comprises exons 1 through 17 of approximately 5.5 kb and having a restriction map for exons 2 through 11, as shown in Figure 8.

According to a further aspect of the invention, exons 1 through 17 have a restriction map for exons 2 through 16, as shown in Figure 9D.

According to another aspect of the invention, a human gene of the above aspects wherein exons 5 through 16 code for the NAIP protein having an amino acid sequence biologically functionally equivalent to the amino acid sequence of Sequence ID No. 2.

According to another aspect of the invention, the human gene of the above aspects have exons 5 through 16 with a cdna sequence biologically functionally equivalent to the cdna sequence of Sequence ID No. 1.

According to another aspect of the invention, a purified nucleotide sequence comprises genetic DNA, cdna, mRNA, anti-sense DNA or homologous DNA corresponding to

the cDNA sequence of Sequence ID No. 1.

According to another aspect of the invention, a DNA molecule sequence coding for the NAIP protein having sequence ID No. 2.

5 According to another aspect of the invention, a purified DNA sequence consists essentially of DNA Sequence ID No. 1.

According to another aspect of the invention, a purified DNA sequence consists essentially of a DNA
10 sequence coding for amino acid Sequence ID No. 2.

According to another aspect of the invention, a purified DNA sequence comprises at least 18 sequential base of Sequence ID No. 1. DNA probes, PCR primers, DNA hybridization molecules and the like may be provided by
15 using the purified DNA sequence of at least 18 sequential bases.

According to another aspect of the invention, use of the DNA sequences of the above aspects in the construction of a cloning vector or an expression vector.

20 According to another aspect of the invention, NAIP protein encoded by the above DNA sequences.

According to another aspect of the invention, NAIP protein comprising an amino acid sequence biologically equivalent to the amino acid sequence of Sequence ID No.
25 2.

According to another aspect of the invention, NAIP protein consisting essentially of the amino acid sequence of Sequence ID No. 2.

According to another aspect of the invention, NAIP
30 protein fragment comprises at least 15 sequential amino acids of Sequence ID No. 2.

According to another aspect of the invention, use of the above amino acid sequences in the production of hybridomas.

35 According to another aspect of the invention, a method is provided for analyzing a biological sample to determine the presence or absence of a gene encoding NAIP

protein.

The method comprises:

- i) providing a biological sample derived from the SMA containing region q13 of chromosome 5;
- 5 ii) conducting a biological assay to determine presence or absence in the biological sample of at least a member selected from the group consisting of:
 - a) NAIP DNA Sequence ID No. 1, and
 - b) NAIP protein Sequence ID No. 2.

10 DESCRIPTION OF DRAWINGS

The original numbering of exons for the NAIP gene begin with exon 0 and progressed through exon 16. This is identified in drawings as sequence numbering Scheme #1. However, for conventional exon numbering, it is
15 preferable to begin with exon 1 and progress through to exon 17. This is now identified as sequence numbering Scheme #2.

Figure 1: YAC contiguous assay of the SMA gene region. YACs are represented by solid lines. Open
20 triangles represent polymorphic STRS, solid triangles represent STSS, open squares represent single copy probes. The genetically defined SMA interval, CMS-1-SMA-D5S557 and the previous D5S629-SMA-D5S557 interval, are indicated above the YACS.

25 Figure 2: Long range restriction map of the SMA region. Rare cutter sites are indicated above the solid line. A minimal set of markers are indicated below the solid line t corresponds to the pYAC4 tryptophan or left end. u corresponds to the pYAC4 uracil or right end. The
30 genetically defined CMS-1-SMA-D5S557 and the D5S629-SMA-D5S557 interval are estimated at 550 kb and 1.1 Mb respectively.

Figure 3: Amplification of the CATT- I locus. Allele sizes are shown below each lane. (A)
35 Amplification of YACS. G: genomic DNA. (B) Amplification of cosmids derived from the chromosome 5 flow sorted library. The 4 distinct alleles are represented by

cosmids 40G1 (allele 15), 58G12 (allele 12), 192F7 (allele 10) and 250B6 (allele 7).

Figure 4: A representative subset of mapped cosmids from our contiguous array. Vertical lines above the
5 solid line are the positions of *EcoRI* sites. Open triangles represent polymorphic STRS, filled triangles represent STSS, filled squares represent single copy probes and open squares represent transcribed sequences. The STRs which demonstrate strong linkage disequilibrium
10 with Type I SMA are indicated by stars. Cosmids IG3 and IB9 are from the YAC 76CI cosmid library.

Figure 5: Sequence duplication in the SMA region identified by p151.2. Hybridization of YACs with (A) the 700 bp fragment and (C) the 500 bp fragment. YACs are
15 arranged from left to right, centromeric to telomeric. Hybridization of cosmids with (B) the 700 bp fragment and (D) the 500 bp fragment. (B) The 12 kb fragment is detected in the cosmids however the 20 kb fragment is not present. The 2.5 kb and 600 bp fragments detected in 3B3
20 and IEI respectively are end fragments. (D) Only the 3 kb fragment is detected in the cosmids. Note the absence of the 20 kb band in 24D6 in (A) but its presence in (C). The 700 bp fragment may be deleted in 24D6.

Figure 6: Degree of linkage disequilibrium observed
25 between Type I SMA and various polymorphic 5q13.1 markers giving a disequilibrium peak at 40G1.

Figure 7: A PAC contiguous array containing the CATT region comprised of nine clones and extending approximately 400 kb. The 2.2 kb transcript referred to
30 as GA1 is shown.

Figure 8: Structural organization of the SMA gene. The exons are represented by black boxes and numbered above. The positions of restriction sites are shown: B, BamHI; E, *EcoRI*; N, NotI, Exons 4 and 5 (Scheme #1) or
35 Exons 5 and 6 of Scheme #2 are frequently deleted in all types of SMA.

Figure 9 is a single page alignment of the information of Figures 6, 1, 7 and 8, respectively. Figure 9(A) is a correlation of the degree of linkage disequilibrium observed in type I SMA families with the disease phenotype and six 5q13.1 markers with the physical map. The SMA containing interval defined by the key recombinations described in the text is shown. Note the proximity of the recombinant peak with the centromeric end of the YAC and PAC contigs, STSs Figure 9(B) is a YAC contiguous array covering the SMA region of 5q13.1. For both YAC and PAC contigs, STSs are denoted by solid triangles, polymorphic tandem repeat polymorphisms by empty triangles, single copy clones by solid squares. Note that our physical map places the CMS sub locus containing allele 9, marked with an asterisk observed to the other CMS subloci, while the reverse was believe, the variation that exists in this region of 5q13.1.

Figure 9(C) is a PAC contiguous array covering the SMA region of 5q13.1. Figure 9(D) is the gene structure of NAIP as provided in more detail in Figure 8.

Figure 10: Exon content of PAC, fetal brain cDNA clones from non-SMA individuals and RT-PCR clones from SMA affected individuals. E158 refers to the deletion of a glutamate residue. The RT-PCR products was only performed between exons 13 and 4 (Scheme #2); additional, undetected deletions may exist outside of this region.

Figure 11: Structure of intact and internally deleted/truncated versions of the NAIP gene as found in the indicated PACs. In Figure 11A, exons under Scheme #2 are marked as numbered black boxes. N refers to NotI sites, B to BamHI and E to EcoRI sites. The EcoRV clone that detects the 3 and 9.4 kb EcoRI bands referred to in the text is denoted by EV in Figure 11B. The 4.8 kb

EcoRI/BamHI band deleted in Figure 14 is also depicted. The 6 kb region containing exons 5 and 6 (Scheme #2) and the 23 kb BamHI fragment resulting from this deletion are both shown in Figures 11C and 11D. The location of
5 primers utilized to identify deletions of exon 5 and 6 as well as those that identify the truncated fragment in the deleted NAIP gene are shown above the NAIP structure.

Figure 12: Intron/exon splice sequences of the NAIP gene.

10 Figure 13: Northern blot of adult tissues probed with exon 13 (Scheme #2) of the NAIP locus. Tissues are as marked and the filter were washed at 50°C, 0.2X SSC and exposed for 4 days. Bands can seen in liver and placenta in the 6-7kb range.

15 Figure 14: Pedigree and Southern blot analysis of consanguineous French-Canadian type III SMA families. Upper panel: probing of a filter containing BamHI/EcoRI digested genomic DNA with a cDNA probe encompassing exons 2 through 9 (Scheme #2) of NAIP reveals the loss of the
20 4.8 kb fragment that contains exons 5 and 6 (Scheme #2) in all affected individuals resulting in an in-frame deletion. All others, save for the homozygous normal sister and brother show half dosage for this band. The lower panel shows a BamHI digest of the same family. In
25 affected individuals two superimposed 14.5 kb contiguous fragments have sustained the 6 kb deletion of sequence containing a BamHI site resulting in the generation of a 23 kb band (see Figure 11). Note the existence of the 23 kb BamHI band in all individuals in the pedigree in
30 keeping with its general dispersion in the population. Similarly, the 9.6 kb BamHI band representing the deletion of exons 1 through 6 (Scheme #2) which is contained in PAC 238D12 and depicted in Figure 11 can be seen in all individuals including non-SMA carriers.

35 Figure 15: Results of PCR amplification in type 3 families 21470 and 24561 using primers 1864 and 1863 which amplify exon 5 (Scheme #2). The reactions were

5 multiplexed with exon 13 (Scheme #2) primers 1258 and 1343 to rule out PCR failure obscuring the results. Failure of amplification in keeping with the homozygous absence of exon 5 (Scheme #2) can be seen to co-segregate with the disease phenotype.

Figure 16: RT-PCR amplification of RNA from SMA and non-SMA tissues. The letter n refers to RNA from non-SMA tissue and a to RNA from SMA affected tissue. The tissue source is shown above each panel. Lym refers to lymphoblast and fib to fibroblast. All samples were from type 1 SMA patients with the exception of a5 which is from an affected member of the consanguineous type 3 SMA family 24561 shown in Figure 15.

RNA was reverse transcribed from exon 13 (Scheme #2). Primary PCR of products shown in panels A and B was with exon 1 primer 1884 and exon 13 primers 1285 or 1974 and those in panel C with exon 6 primer 1919 and exon 13 primer 1285. Secondary PCR reactions for panel A used exon 4 primer 1886 and exon 13 primer 1974; for panel B, exon 5 primer 1864 and exon 11 primer 1979 and for panel C, exon 9 primer 1844 and exon 13 primer 1974.

Failure or amplification of reduced products can be seen in panel A for spinal cord and lymphoblast tissue for samples a2, a3, a4, a5, a6 and a7. Panel B also shows amplification of reduced size bands in a2 and a3, and in a7 a larger product in keeping with an insertion. Panel C shows reduced band size in keeping with deletions of exons 11 and 12 (Scheme #2) in a2, a3, a9 and a11.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless indicated otherwise, reference to exons in this detailed description of the invention will be based on exon numbering Scheme #2.

Throughout the specification, various letter abbreviations will be used to identify various components or techniques. The following glossary is provided to reference these items.

	CTR	-	complex tandem repeat
	DNA	-	deoxyribonucleic acid
	PCR	-	polymerase chain reaction
	PFGE	-	pulsed field gel electrophoresis
5	PAC	-	P1 artificial chromosome
	RNA	-	ribonucleic acid
	RT-PCR	-	reverse transcriptase-polymerase chain reaction
	STR	-	simple tandem repeat
10	STS	-	sequence tag site
	YAC	-	yeast artificial chromosome

This invention is directed to the identification, location and sequence characteristics of a gene which encodes Neuronal Apoptosis Inhibitor Protein (NAIP). We have established that mutations in this gene are causative of the previously discussed types I, II and III of Spinal Muscular Atrophies (SMA). It is believed that mutations in this gene result in the lack in the production of normal NAIP protein which is believed to be physiologically involved in the normal human process of maintaining neurological cells and preventing their early death common to affected individuals. The subject gene maps to the SMA containing region of chromosome 5q13.1. Unless indicated otherwise, reference to exons in this detailed description of the invention will be based on exon numbering Scheme #2. The gene comprises exons 1 through 17 of approximately 5.5 kb and has a restriction map for exons 2 through 11, as shown in Figure 8. An updated restriction map for exons 2 through 16 is provided in Figures 9D and 11A. As is appreciated, the gene is considerably longer than the sequence for exons 1 through 17. Considerable intron information exists between the exons which has not yet been sequenced. From the standpoint of diagnosing SMA, the sequence information of exons 1 through 17 is very valuable. The normal sequence is provided in Table 4, as well as being listed under Sequence ID No. 1. Any genetic mutation,

that is, changes in the DNA sequence, whether they be due to deletion, entire absence of gene substitution or polymorphisms and the like, are or can be causative of the disease. The most common mutations are thought to

5 be:

- i) deletion of exons 5, 6 of the gene; or
- ii) absence or marked reduction in the copy number of this gene in the chromosome 5 can be causative, if the remaining genes are defective.

10 Any form of biological assay may be employed to diagnose a person's susceptibility to SMA by virtue of conducting a biological assay to determine the normal sequence or absence or presence of mutations in the normal sequence. Such biological assays may include DNA
15 hybridization by use of DNA probes and the like, restriction enzyme analysis, PCR amplification of the relevant portions of the sequence, messenger RNA detection and DNA sequencing of the relevant portions of the sequence, as isolated from chromosome 5 of the human
20 biological sample. It is appreciated that a variety of the above generally identified biological assay procedures may be conducted where the preferred techniques are as follows:

SMA diagnoses will be conducted in two ways.

25 Initially, the genome of the human at risk will be assayed for the absence of NAIP exons 5 and 6. These exons are found to be absent with a frequency of .05% in the general population and 50% in Type 1 SMA. The second approach will be to assess the number of copies of the
30 NAIP gene in the individuals being tested. We have observed that there is a general depletion of both deleted and intact forms of the NAIP gene, in individuals with SMA. By using a densitometric approach to assess the number of gene copies, an accurate assessment of the
35 risk having SMA can be established. The best correlation is observed for exons 2 through 4 and exon 13.

In practical terms, the two steps outlined above will be conducted in the following manner:

- (i) two concurrent PCR reactions will be carried out upon the same aliquot of DNA (0.1 micrograms) from the human in question. One primer pair will map into exons 5 and 6 (e.g. primers 1863 Sequence ID No. 7 and 1864 Sequence ID No. 8) and one pair will be homologous to a region outside of exons 5 and 6 (primers 1343 Sequence ID No. 5 and 1258 Sequence ID No. 4). The latter reaction will be performed to ensure that the PCR is functioning. Two additional controls will be (i) PCR performed on genomic DNA known to contain exons 5 and 6 employing the appropriate primers to ensure that this particular reaction is working, (ii) negative controls using water as a template to ensure absence of contamination. All PCR products will be placed in an agarose gel, separated electrophoretically and analyzed visually.
- (ii) Densitometric assessment of SMA risk will be carried out by using PCR primers tagged with fluorescent dyes. PCR reactions employing primers for exons 2 through 4, exons 13 as well as exons 5, 6 and exons 11, 12 will be performed on genomic DNA from the individual being assessed. PCR products will be separated electrophoretically on a gel and the intensity of the individual bands assessed fluorometrically. These values will be correlated with normative values and SMA risk thus ascertained.

It is apparent that one's level of NAIP correlates with the risk for other neurodegenerative disorders such as amyotrophic lateral sclerosis and Alzheimers. Consequently, the tests outlined above serve as predictors of risk for these disorders as well. As is described in more detail in the section under heading *Baculoviral IAPs*, the NAIP protein has significant homology with proteins for inhibiting cell apoptosis. Hence, any neurodegenerative disease which is based on

13

neuronal cell apoptosis can now be predicted by use of the DNA sequence information of the NAIP gene. Such neuronal cell apoptosis is most likely linked to mutations in the NAIP gene similar to the mutations associated with SMA or other mutations in the gene which affect the biological activity of the NAIP protein inhibiting neuronal apoptosis.

As to mRNA detection we propose the following: RT-PCR is a rapid technique for the analysis of RNA transcripts which is a crucial part of several molecular biology applications. This method is much more sensitive and efficient than traditional Northern blot, RNA dot/slot blots, and in situ hybridization assays. The sensitivity of such a technique allows one to study RNA amounts of low abundance or RNA isolated from small transcripts of cells. In addition, an entire panel of transcripts can be analyzed simultaneously.

Protocol Summary: RNA is first isolated from tissues or cells and then is used as a template for reverse transcription to complementary DNA (cDNA). The reverse transcription (RNA-directed synthesis of DNA), is catalyzed by the enzyme reverse transcriptase. The cDNA is then used as the template for PCR using primers designed to amplify a selected cDNA region. Following PCR, the product is analyzed by agarose gel electrophoresis. The amplified cDNA is identified by the size of the PCR product which is predicted from knowledge of the cDNA nucleotide sequence. The PCR product can be further validated by restriction digestion, hybridization or nucleotide sequencing.

Enzymatic Amplification of RNA by PCR (RT-PCR)
This method is used to enzymatically amplify RNA using PCR.

Detailed Protocol: First the primer is annealed to the RNA. The RNA and cDNA primer are coprecipitated by adding together poly(A)⁺RNA, cDNA primer, and water.

Sodium acetate is added and ethanol. This is precipitated overnight over -20°C . The pellet is collected after microcentrifugation. The pellet is washed with ethanol. Then water, Tris-HCl, and KCl are added and the mixture is heated to 90°C and then cooled slowly to 67°C . Microcentrifuge and incubate 3 hours at 52°C . This final annealing temperature may be adjusted according to base composition of primer. Alternatively, the primer can be annealed to the RNA by mixing poly(A)⁺RNA, cDNA primer, and water. This mixture is heated 3 to 15 minutes at 65°C . To the cooled mixture, add reverse transcriptase buffer.

The cDNA is now synthesized. Add reverse transcriptase buffer and AMV reverse transcriptase. This is mixed and incubated 1 hour at 42°C (depending on the base composition of primer and RNA). Add Tris-Cl/EDTA, mix then buffered phenol and vortex. Microcentrifuge and add chloroform to the aqueous phase and vortex. Microcentrifuge. Add sodium acetate and ethanol to aqueous phase. Mix and precipitate overnight at -20°C . Microcentrifuge, dry pellet, and resuspend in water.

The cDNA is then amplified by PCR. The mixture contains prepared cDNA, amplification, dNTP mix, amplification buffer, and water. Usually one of the amplification primers is the same as cDNA primer. If a different amplification primer is used, the cDNA primer should be removed from the cDNA reaction. The reaction mixture is then heated 2 minutes at 94°C , and microcentrifuged to collect condensate. Add Taq DNA polymerase, mix, centrifuge, overlay with mineral oil. Set up amplification cycles. The number of cycles is varied depending upon the abundance of RNA. Forty cycles are usually sufficient. The products are then analyzed by gel electrophoresis in agarose or nondenaturing polyacrylamide gels. The cDNA can also be introduced directly into the amplification step.

In referencing the gene, its cDNA sequences, other DNA sequences and RNA sequences, it is understood that any specifically referenced sequence includes any and all biologically functional equivalence thereof. Similarly, with listed protein sequences, it is understood that such terminology includes any and all biologically functional equivalence thereof insofar as the intended purpose is concerned. In the above identified biological assays it is understood that the full length or partial length sequences of the DNA or protein may be used. Generally it is contemplated that at least 18 sequential bases of the DNA sequence are useful as hybridization probes, PCR primers and the like. Similarly, with protein sequences, at least 15 sequential amino acid sequences may be correspondingly useful in developing protein receptors such as monoclonal antibodies. Such monoclonal antibodies may be made in accordance with the standard techniques by developing hybridomas for producing monoclonals specific to certain antigenic determinants of the protein structure.

With reference to Table 4, it would appear that in view of the significant homology of exons 5, 6, 7, 8, 9, 10 11 and 12 with the IAP domains, such homology may well mean that any deletions or other forms of mutations in these exons may result in the carrier being susceptible to the disease. For example, this is evidenced by the deletion of exons 5 and 6 in low copy numbers in humans being causative of the disease. Hence, any of the sequence information in this region of the gene will be important from a diagnosis standpoint so that any sequential 18 bases of DNA or 15 sequential amino acid residues in this region may be relied on in the diagnosis of SMA in suspected humans. It is of course also understood that other forms of deletions, mutations, polymorphisms and the like in other regions of the gene may be causative of the disease or may be used for other purposes in conjunction with disease analysis, prognosis

and perhaps treatment.

Although the restriction maps are useful in identifying the characterizing features of the subject gene the specific cDNA sequence of exons 1 through 17 has
5 been provided in sequence ID No. 1. The encoding portion of the sequence commences at the ATG codon of base 396 of exon 5. The encoding portion ends at the stop codon TAA of exon 16 at base position 4092. Exons 1 through 4 are at the 5' untranslated region and exon 17 is at the
10 3'untranslated region. As with some genetic related diseases, mutations or polymorphism in the untranslated regions may as well be causative of the disease so that sequence portions in the form of probes and the like in regions other than the region of significant IAP homology
15 may be valuable in the diagnosis of SMA. It is also understood that the sequence information of sequence ID No. 1 may be used in the construction of suitable cloning vectors for purposes of producing multiple copies of the gene or expression vectors for purposes of transfecting a
20 host to produce significant quantities by recombinant techniques of the NAIP protein. Sections or fragments or full-length sequence information may be used in the construction of the cloning vectors or expression vectors depending upon the end use of such vectors. With this
25 understanding, the details in respect of the identification of the SMA disease gene its characteristics, the corresponding protein sequence and their uses in diagnosis are explained.

A YAC contig of the Spinal Muscular Atrophy (SMA)
30 disease gene region along chromosome 5q13 was produced which incorporated the D5S435-D5S112 interval and encompassed 4 Megabases. The CATT-40G1 subloci on the cosmid array showed significant linkage disequilibrium with Spinal Muscular Atrophy indicating close proximity
35 to the gene. However, delineation of the precise region containing the SMA gene was not possible based on this information alone. A PAC contiguous array containing the

CATT region comprised of 9 clones and extending approximately 400 kb was constructed. The genetic analysis combined with the physical mapping data indicated that the 154 kb PAC clone 125D9 (Figure 7) which contained the CMS allele 9 and the 40G1 CATT sublocus had a good probability for containing the SMA locus. Through further analysis as will be described, PAC 125D9 was found to contain the gene encoding neuronal apoptosis inhibitor protein.

5 PYAC (yeast artificial chromosome plasmids) allow direct cloning into yeast of contiguous stretches of DNA ≤ 400 kb. Circular PYAC plasmids (without inserts) can replicate in *E. coli*. In vitro digestion of PYAC, 10 ligated to exogenous DNA, and direct transformation of the subsequent linear molecules (with telomeric sequences at each termini) into yeast generates a library that can be screened by standard techniques.

15 Large YAC constructs are as stable as natural chromosomes. They are good vectors for the construction of libraries from complex genomes such as the human genome. In addition, sequences which are unclonable in *E. coli* cosmid and lambda vectors are successfully cloned in YAC vectors.

20 YAC vectors are normally propagated in bacteria as circular plasmids. Restriction enzyme target sites are arranged to produce two arms upon digestion, each of which contains a different selectable marker and terminates at one end in a telomere, the other in a blunt end. In addition, one of the arms contains an ARS element. The two arms are purified away from a linking fragment and ligated with donor DNA fragmented so as to leave blunt ends. The ligation mixture is used to transform yeast cells, and the selection conditions are such as to require the presence of both arms, the insert 30 interrupts a third selectable marker which allows non-recombinant structures to be recognized.

Construction of YAC Contig

YAC clones were isolated from three libraries, constructed at the National Centers of Excellence (NCE, Toronto), the Imperial Cancer Research Fund (ICRF, London) (Larin et al., 1991) and the Centre d'Etude du Polymorphisme Humaine (CEPH, Paris) (Albertson et al., 1990), all of which were prepared from partial *EcoRI* digests of total DNA ligated into the YAC vector pYAC4. ICRF YAC clones were identified by probing library filters with 5q13.1 probes. YAC DNA from the NCE library was screened by PCR amplification, electrophoresed, immobilized onto Southern blots and hybridized with the radiolabelled STS product to identify positives. Numerous positives were obtained repeatedly in both the initial round of PCR of pooled plates, and the second round with the plate(s) thought to contain the clone of interest many of which proved to be false positives. The number of false positives obtained, which appeared to be primer dependent, was reduced by radiolabelling PCR products and resolving these on 6% polyacrylamide gels. The true positives could then be sized accurately without interference from spurious products.

Yeast strains with YACs positive for 5q13.1 STSs were grown on selective plates and examined for stability in the following manner: 4 colonies of each were grown for preparation in agarose blocks, yeast chromosomal DNA was separated by pulsed field gel electrophoresis and transferred to filters and the size and number of YAC clones contained within each yeast colony was determined by hybridization with radiolabelled total human genomic DNA. Positive clones were confirmed either by hybridization or PCR amplification with the original probe. Only YAC 24D6-2 contained some colonies with more than one YAC.

YAC end clones and inter-Alu products were isolated by vector-Alu PCR and inter-Alu PCR respectively. The location of these products within 5q11-13 was confirmed

by hybridization to Southern filters of the somatic cell hybrids HHW105 (Dana et al., 1982), containing the entire chromosome 5, and HHW1064 (Gilliam et al., 1989), a derivative containing chromosome 5 with a deletion at 5q11.2-13.3. Many of these probes demonstrated hybridization profiles indicative of locations both within the 5q11-13 region and elsewhere on chromosome 5. In some cases primers specific for the ends of each YAC were generated by vector-Alu-PCR. The mapping of each YAC isolated by PCR amplification of DNA from the somatic cell hybrids HHW105 and HHW1064. In a few cases it was found that a primer pair contained a product from both HHW1064 and HHW105 were positive. Formulation of new STS primers resulted in the amplification of new STS products specific to the 5q11-13 region. End clone hybridization and STS analysis performed on all YACs confirmed the orientation and location of each YAC. The assembly of a contiguous array of YACs covering the SMA interval was initiated from two markers which flank SMA; D5S125 (Mankoo et al., 1991), which lies centromeric to D5S435 and the more telomeric marker D5S112 (Lien et al., 1991) (see Figure 1). Six YACs were identified in the ICRF library by the telomeric marker pJK53 (D5S112). One of these YACs, D06100, was shown to extend the furthest centromerically based on end clone STS analysis. The centromeric end of this YAC identified two YACs from the NCE library, 1281 and 1284. YACs positive for the D5S125 or D5S435 markers were not found in the ICRF or NCE library thus the CEPH library was screened, from which clones containing D5S435 were isolated. A microsatellite polymorphism mapping into the center of the gap, CATT-1 (Burghes et al., 1994), was utilized to detect three YACs, 24D6-2, 27H5 and 33H10. These YACs were shown to be linked to both the centromeric and the telomeric YACs (1281, 1284) by STS

analysis. Internal YAC products generated by AluPCR were utilized to probe all YACs establishing the degree of overlap. STS sequences (Kleyn et al., 1993) mapping between JK348 and D5S112 were utilized to confirm the degree of overlap and the orientation of YACs in the contig. Concurrently the order of each STS along 5q13 was confirmed. In all a total of 14 YACs were identified, anchored by the genetic markers D5S435, D5S629, CMS-1, CATT-1, D5F153, D5F149, D5F150, D5F151, D5S557 and D5S112.

Long Range Restriction Map and Estimation of Long range Physical Distance

A restriction map of the critical SMA region was constructed from the STS Y116U (Kleyn et al., 1993), approximately 100 kb proximal to D5S629, to the STS Y107U (Kleyn et al., 1993), which lies approximately 500 kb distal to D5S557 (see Figure 2). In order to detect any possibility of deletions or rearrangements in our YACs, additional YACs isolated from the CEPH library (Kleyn et al., 1993), mapping within this region were included in the analysis. YACs 24D62, 27H5, 33H10, 155H11, 76C1, 235B7, 184H2, 428C5, and 81B11 (Kleyn et al., 1993) were partially digested utilizing the rare cutter restriction endonucleases *NotI*, *BssHII*, *SfiI*, and *RsrI*. Southern blots of the Pulse Field Gel Electrophoresis (PFGE) separated restriction products were hybridized with YAC left arm and right arm specific probes which revealed the positions of cleavage sites from both ends of each YAC. The orientation and overlap of the YACs had been previously determined based on STS analysis, therefore the position of the rare cutter sites among the overlapping YACs were compared. By aligning the overlapping YACs at their common rare cutter sites, the degree of overlap could be more precisely determined. The long range restriction map of the overlapping YACs derived from different sources was mostly in agreement

with the exception of 33H10 and 428C5. 428C5 has previously been documented to contain a deletion (Kleyn et al., 1993), evident by comparison of its STS content and its size of only 300 kb, indicating that it lies further centromeric than its placement in Figure 2. YAC 33H10, based on STS analysis contains an internal deletion and YAC 155H11 is chimeric at its telomeric end therefore rare cutter sites at the telomeric end of the map which could not be confirmed were not included. The results indicate the distance from the centromeric boundary D5S435 to the telomeric boundary D5S557 to be 1.4Mb in marked contrast to 400 kb as previously reported (Francis et al., 1993) but in agreement with one other estimate (Wirth et al., 1993). Furthermore, the D5S629-D5S557 interval can be estimated at 1.1 Mb and the distance of the genetically defined CMS1-SMA-D5S557 interval is approximately 550kb.

Cosmid Contig Assembly from the Chromosome 5 Library

Although the isolation of cosmids utilizing whole YACs as probes could be an expeditious method of constructing a cosmid contig, in this case the presence of chromosome 5 specific repeats would likely result in the isolation of cosmids mapping elsewhere on chromosome 5. A directed cosmid walking strategy was thus adopted. The CATT-1 STR, which has been shown by irradiation hybrid analysis to map approximately midway between the two flanking markers D5S435 and D5S351 (Hudson et al., 1992), was utilized as the initiation point for the construction of a cosmid clone array. The complex pattern of amplification seen on genomic DNA, with two to eight alleles per individual (see Figure 3), suggested a variable number of copies or loci of the CATT-1 sequence in this region. Thirty CATT-1 positive cosmids were identified which upon PCR analysis were seen to contain one of four distinct alleles (see Figure 3). As the cosmid library was derived from a monochromosomal source,

this confirmed that the CATT STR exists at least in four locations, which we refer to as subloci. These subloci are referred to as CATT-40G1, CATT-192F7, CATT-58G12 and CATT250B6-based on the cosmid addresses of the first 5 cosmids identified containing alleles of 12, 19, 15 and 20 cytosine adenosine (CA) dinucleotides respectively. Bi-directional walking was initiated from these 4 cosmid subloci. Positive hybridization was observed for cosmid 250B6 with one end of 58G12 and for 192F7 with the other 10 end resulting in the ordering of cen-192F7-58G12-250B6-tel (Figure 4). All cosmids which contained the CATT-192F7 allele were mapped to this location based on the size of their CATT-1 allele and their restriction enzyme profiles. As shown in Figure 4 the CATT-192F7 sublocus 15 is telomeric to the STR CMS-1, which itself lies telomeric to the CATT-40G1 sublocus.

Due to the presence of chromosome 5 specific repetitive sequences, resulting in the identification of cosmids from another region of chromosome 5, the 20 integrity of the contig was verified with each step taken. Cosmid end clones generated by vector-Alu-PCR were hybridized to somatic cell hybrid panels as described above. As repetitive sequences which map solely to the region of chromosome 5 that is deleted in 25 the hybrid cell line HHW1064 have been observed, cosmids identified by end products which did not hybridize to HHW1064 were analyzed further. Proof of overlap was shown by hybridization of end clones, single copy probe hybridization, STS content, and restriction enzyme 30 profile comparison. Cosmids identified by end clones — which hybridized to HHW1064 were eliminated and walking was continued by utilizing a different inter-Alu product from the clone of origin, which was verified in the same manner. Cosmid sizes were calculated by the addition of 35 EcoRI restriction fragments and the extent of overlap was determined by the addition of those fragments in common.

Cosmid Contig Assembly of YAC 76C1 Cosmids

As extension of the cosmid contiguous array was prevented by the presence of chromosome 5 specific repeats, a 5X cosmid library was produced from YAC 76C1.

- 5 The STSs CATT- 1, CMS-1, Y122T (Kleyn et al., 1993), Y97T (Kleyn et al., 1993) and Y98T (Kleyn et al., 1993), which are distributed along the YAC were utilized to identify cosmids to assemble the contig. As well, the previously developed markers, pZY8, pL7, pGA-1, p15.1, p402.1, 10 p2281.8 and β -glucuronidase (Oshima et al., 1987) (Table 2, Figure 4) from the established cosmid contig were hybridized to the library providing an effective method of ordering the cosmids. Cosmids demonstrating irregular hybridization patterns and thought to contain deletions 15 and/or rearrangements were excluded.

- The STS Y98T identified three cosmids including one previously identified by the probe p2281.8, derived from a chromosome 5 library clone, 228C8, also containing the STS Y98T. An end product of this cosmid hybridized to 20 ten cosmids. Concurrently, an end fragment of a CATT40G1 sublocus was shown to hybridize to four of these ten cosmids thus linking CATT-40G1 and CMS-1 with the more centromeric STS Y98T (Figure 4). We were unable to identify any clones containing the YAC end STS Y97T.
- 25 Filter hybridization and STS mapping experiments indicated a second more telomeric location of the CATT40G1 sublocus. A duplication of this sublocus would agree with genotype data in our SMA kindreds (McLean et al., in press).

- 30 An *EcoRI* restriction map was generated utilizing a minimal set of cosmids necessary to span the region. To ensure the reliability of the contig, we sought to integrate it with the contig constructed from the chromosome 5 specific library. Concordance of the 35 contigs was evident by comparison of the restriction maps, the position of probes and STSs on the map and Alu-PCR fingerprinting. In this manner the size of the

contig was estimated to be 210 kb. A directed walking strategy has thus resulted in the generation of a single contiguous set of cosmids containing the CATT-1 cluster of subloci with known centromere/telomere orientation.

5

Duplications/Deletions

Several lines of evidence suggested the presence of genomic sequence duplications within our cosmid array. We provide evidence for the duplication of the CATT-40G1 sublocus in cosmids derived from a single chromosome 5. A centromeric location for this sublocus established as the CATT-40G1 sublocus was found to be contiguous with the STSs Y122T, Y88T and CMS-1 in several cosmids, and the centromeric YAC 428C5 is positive for probes isolated from the CATT-40G1 containing cosmids. Although YAC 428C5 does not contain the CATT40G1 sublocus upon PCR amplification, this may be explained either by a null allele in the chromosome from which the YAC was derived or a deletion in the YAC. We have previously observed null alleles in individuals at distinct CATT-1 subloci. A second more telomeric location of CATT-40G1 was determined by the hybridization to CATT40G1 cosmids of the probes pGA- 1, pL7, and pZY8 all of which bind the more telomeric YACs 33H10, 24D62. The hybridization of p402.1, derived from cosmid 40G1, to cosmids at both locations would indicate that the duplication is not restricted to the CATT-40G1 subloci and likely encompasses a larger region. Southern blot analysis revealed distinct profiles of cosmids for the two locations however common bands were detected by Alu-PCR fingerprinting supporting a duplication.

Correlation of our YAC contig with the cosmid contig revealed that YACs 76C1, 81B11, and 27H5 span the 150 kb CATT region of 5q13. Despite this, CATT-1 genotyping of these YACs revealed only one allele size, raising the possibility that the chromosomes from which these YACs were derived (4 in all) contain null alleles at their

remaining CATT-1 subloci. Our experience, however, with CATT linkage analysis of SMA families indicated that such a scenario is highly unlikely as none of the approximately 300 individuals genotyped had fewer than 2
5 alleles. We consequently believe it is more likely that these CATT subloci are unstable and have been deleted during YAC construction and/or propagation.

Sequence comparison between the CATT-1 and D5F153 primer sequences indicated that these two STRs were
10 similar and possibly the same as one primer is identical and the other primer sequences overlap by eight nucleotides. However, the centromeric YACs 428C5, 232F12, 235B7, 184H2, and the telomeric YACs 12H1, 155H11, 269A6 which were CATT-1 negative yielded D5F153
15 amplification products indicating that CATT-1 may be a derivative of D5F153. These data, in combination with D5F153 analyses of the cosmid contig, which contains three D5F153 loci (Figure 4), indicated that at least five D5F153 subloci exist.

20 In addition to the CATT-1 and D5F153 STRs, the STRs CMS-1 and D5F150 were present in a variable number of copies per chromosome 5. STS analysis localized CMS-1 to YACs 428C5, 76C1, 81B11 and 27H5 with allele sizes of 5, 4, 4 and 3, and 4 respectively. PCR amplification of
25 genomic DNA revealed up to four alleles per individual indicating as many as two copies per chromosome. D5F150 was present at two locations within the cosmid array yet only one location was detected in the YAC contig. D5F151 was not detected within our cosmid array nevertheless it
30 was placed at the centromeric end of YAC 33H10, which encompasses the cosmid array, based on the positive amplification of YAC 428C5. One location of D5F149 was detected on both our cosmid and YAC clones. Our data suggested, as with CATT-1, the existence of null alleles
35 and/or instability of the CMS-1, D5F150, D5F151, D5F149 sequences in YACS.

A deletion event was observed in hybridization with an 800 bp *EcoRI* fragment isolated as a single copy probe from the CATT-40G1 containing cosmid 234A1 from the chromosome 5 specific cosmid library. Probing of YAC DNA failed to detect this fragment in any of our YACs. Hybridization to genomic DNA of several individuals did not identify any deletion events thus this sequence may be susceptible to instability in the YACS. Sequencing of this fragment did not reveal any exons or coding region.

Further evidence of sequence duplication in the SMA region was identified with a 1.2 kb internal *Alu*-PCR product (p151.2) from cosmid 15F8 (Figure 4). The probe identified three *EcoRI* fragments in YAC clones 76C1, 81B11 and 27H5 (20 kb, 12 kb and 3 kb) but only one in 33H10 and 24D6 (20 kb) and one in 428C5 (12 kb). An internal *EcoRI* site divided this marker into 500 bp and 700 bp probes. The larger probe identified the 12 kb and 20 kb fragments while the smaller probe identified the 3 kb and 20 kb fragments (Figure 5). We ruled out instability of this sequence in YACs as they are from different libraries and the hybridization patterns reflected their physical location. The 12 kb and 3 kb fragments were localized on the *EcoRI* restriction map, however we were unable to position the 20 kb fragment. Taken together these findings suggest the 12 kb and 3 kb lie in tandem with a centromeric/telomeric orientation respectively. A location of the 20 kb fragment distal to our contiguous array of cosmids may be inferred from the data. The duplication was confirmed by hybridization to genomic DNA digests revealing all three fragment sizes.

YAC Contig and Cosmid Contig Characteristics

We established a YAC contig of the SMA disease gene region, incorporating the D5S435-D5S112 interval and encompassing 4 Mb. Orientation of the contig along 5q13 was confirmed by analysis of seven genetic markers and STSs in combination with PFGE analysis. The long range

restriction map revealed neither major deletions nor rearrangements among the YACs within our contig, and was utilized to refine the estimates of the size of the contig. Our YAC map establishes physical linkage of the markers D5S629, D5F153, D5F151, D5F150, D5F149, CMS-1, CATT-1 and D5S557 to a 1.1 Mb region, a region of the genome characterized by low copy repetitive sequences and multilocus STRS. Furthermore, we estimated the new genetically defined CMS1-SMA-D5S557 to be 550 kb.

Estimates of the physical distance of the D5S435-D5S557 interval ranging from 400 kb (Francis et al., 1993) to 1.4 Mb (Wirth et al., 1993) have been reported. In contrast to these studies our estimation of 1.4 Mb for the D5S435-SMA-D5S557 interval and 550kb for the CMS11-SMA-D5S557 interval, employs clones derived from three sources, comprised of 6 chromosomes. Moreover, the determination of both the size of clones and the position of rare cutter sites has enabled us to determine more precisely the extent of overlap of the YACs and the size of the contig providing a reliable estimation.

We also assembled a single contiguous array of cosmid clones derived from both a chromosome 5 specific library and a YAC (76C1) specific library in conjunction with a restriction map of the CMS-1/CATT-1/D5F153/D5F150/D5F149 region encompassing 210 kb. The repetitive sequences prevented extension of the cosmid contig when utilizing a chromosome 5 specific library necessitating construction of a cosmid library YAC 76C1 in the critical region. The contiguous cosmid array was constructed by a directed walking strategy with validation of cosmid overlap established by restriction fragment enzyme overlap, Alu fingerprinting, and analyses involving STSs, cosmid end clones and single copy probes.

Physical and genetic mapping analyses revealed a complex region of genomic DNA comprising duplications and the presence of repetitive sequences. Genotyping of genomic DNA with complex STRs from this region revealed

the presence of a polymorphic number of bands ranging as high as eight per individual. This suggested the presence of multiple copies, or subloci, for the STRs CATT-1, CMS-1, D5F153, D5F150. Our physical mapping data confirmed the presence of these subloci except in the case of D5F151 and D5F149 which revealed only one location. Four of the CATT-1 subloci map to our cosmid array within a 140 kb region; at least one of these subloci, CATT-40G1, is duplicated. D5F153 and CATT-1 are related STRs which appear to have diverged from a common ancestor. We had localized one CMS-1 sublocus to our cosmid array, however, we were unable to determine from our data whether other subloci exist on other chromosomes within this 200 kb interval, as the chromosomes from which the YAC/cosmid libraries were derived may either contain null alleles at the remaining subloci or have sustained deletions.

The CATT-1, D5F153, D5F150 and D5F149 STR, although present in multiple copies on chromosomes in the population were observed as single sublocus markers on all YACS, as evidenced by single allele PCR products for each, suggesting instability and deletion of these sequences. This is supported by the absence in our YACs of an 800 bp fragment, derived from the chromosome 5 cosmid library based contiguous array. Instability of these sequences does not appear to result in large deletions as additional unique sequence probes located between the multiple subloci are retained in the YACs.

In summary, we have produced the first high resolution physical map of the critical SMA region. However, delineation of the precise region which contained the SMA gene was not possible based on this information alone.

Concurrent with our genetic analysis, we constructed a YAC contiguous array employing clones from three different YAC libraries (Roy et al., 1994). A minimal representation from this array, which was correlated with

extensive pulsed field gel electrophoresis (PFGE) analysis, is shown in Figure 9B.

With the initial suggestion of linkage disequilibrium of the general CATT marker and SMA
5 (Burghes et al., 1994), the construction of a cosmid contiguous array incorporating the extended CATT region was undertaken. The presence of extensive and polymorphic genomic repetitive elements mapping both to 5q13 and elsewhere on chromosome 5 interfered with a
10 straightforward assembly of a contiguous array. However, the integrity of the array was established by restriction enzyme analyses, Alu-PCR fingerprinting, STS content determination and nucleic acid hybridization using cosmid end clones and other single copy probes. This resulted
15 in the generation of an array encompassing 220 kb that contained the five CATT subloci contained in a mono-chromosomally derived flow sorted chromosome 5 genomic library (Roy et al., 1994). More recently, a P1 artificial chromosome (PAC, Ioannou et al., 1994)
20 contiguous array containing the CATT region, comprised of 10 clones and extending approximately 550 kb, was constructed (Figure 9C).

Linkage Disequilibrium Analysis

25 A linkage disequilibrium analysis employing 5 complex and simple tandem repeats mapping to the SMA region was conducted. Two of the polymorphisms employed in this analysis were the CATT-40G1 and CATT-192F7 subloci which we mapped to our cosmid array. Specific
30 amplification of the two individual subloci was achieved by constructing primers ending on sequence polymorphisms in the region flanking the CA repeat. A clear linkage disequilibrium peak was observed at the CATT-40G1 sublocus as shown in Figure 6.

PAC Contig Array

Since the 40G1 CATT subloci demonstrated linkage disequilibrium, a PAC contiguous array containing the CATT region was constructed. This PAC contig array
5 comprised 9 clones and extended approximately 400 kb (Figure 7). Our genetic analysis combined with the physical mapping data indicated that the 40G1 CATT subloci marker which showed the greatest disequilibrium with SMA was duplicated and was localized at the extreme
10 centromeric of the critical SMA interval. Consequently the 154 kb PAC clone 125D9 which contained within 10 kb of its centromeric end the SMA interval defining CMS allele 9 and extended telomerically to incorporate the 40G1 CATT sublocus was chosen for further examination.

15 Two genomic libraries were constructed by performing complete and partial (average insert size 5 kb) Sau3A1 on PAC 125D9 and cloning the restricted products into BamHI digested Bluescript plasmids. Genomic sequencing was conducted on both termini of 200 clones from the 5 kb
20 insert partial Sau3A1 library in the manner of (Chen et al., 1993) permitting the construction of contiguous and overlapping genomic clones covering most of the PAC. This proved instrumental in the elucidation of the neuronal apoptosis inhibitor protein gene structure.

25 PAC 125D9 is cleaved into 30 kb centromeric and 125 kb telomeric fragments by a NotI site (which was later shown to bisect exon 7 of the PAC 125D9 at the beginning of the apoptosis inhibitor domain. The NotI PAC fragments were isolated by preparative PFGE and used
30 separately to probe fetal brain cDNA libraries. Physical mapping and sequencing of the NotI site region was also undertaken to assay for the presence of a CpG island, an approach which rapidly detected coding sequences. The PAC 125D9 was also used as a template in an exon trapping
35 system resulting in the identification of the exons contained in the neuronal apoptosis inhibitor protein gene.

The multipronged approach, in addition to the presence of transcripts identified previously by hybridization by clones from the cosmid array (such as, GA1 and L7), resulted in the rapid identification of six
5 cDNA clones contained in neuronal apoptosis inhibitor protein gene. The clones were arranged, where possible, into overlapping arrays. Chimerism was excluded on a number of occasions by detection of co-linearity of the cDNA clone termini with sequences from clones derived
10 from the PAC 125D9 partial Sau3A1 genomic library.

Cloning of Neuronal Apoptosis Inhibitor Protein Gene

In the meantime, a human fetal spinal cord cDNA library was probed with the entire genomic DNA insert of
15 cosmid 250B6 containing one of the 5 CATT subloci. This resulted in a detection of a 2.2 kb transcript referred to as GA1 which location is shown in Figure 7. Further probings of fetal brain libraries with the contiguous cosmid inserts (cosmids 40G1) as well as single copy
20 subclones isolated from such cosmids were undertaken. A number of transcripts were obtained including one termed L7. No coding region was detected for L7 probably due to the fact that a substantial portion of the clone contained unprocessed heteronuclear RNA. However, we
25 later discovered that L7 proved to comprise part of what is believed to be the neuronal apoptosis inhibitor protein gene. Similarly, the GA1 transcript ultimately proved to be exon 13 of the neuronal apoptosis inhibitor protein. Since GA1 was found to contain exons indicating
30 that it was an expressed gene, it was of particular interest. The GA1 transcript which was contained within the PAC clone 125D9 was subsequently extended by further probing in cDNA libraries.

The extended GA1 transcript was compared to other
35 known sequences to reveal that its amino acid sequence had significant homology to the inhibitor apoptosis polypeptides of *Orgyia Pseudotsugata* and *Cydia Pomonella*

viruses (Table 3). This sequence analysis revealed the presence of inhibitor apoptosis protein homology in exons 5 and 6.

The remaining gaps in the cDNA were completed and the final 3' extension was achieved by probing a fetal brain library with two trapped exons. A physical map of the cDNA with overlapping clones was prepared. The entire cDNA sequence is shown in Table 4 and contains sixteen exons. The amino acid sequence starts with methionine which corresponds to the nucleotide triplet ATG. Figure 8 demonstrates the structural organization of the SMA gene.

The cDNA sequence of NAIP shown in Table 4 allows one skilled in the art to develop from this gene, primers, probes and also antibodies against the protein product. The cDNA sequence of Table 4 may be used in recombinant DNA technology to express the sequence in an appropriate host in order to produce the neuronal apoptosis inhibitor protein. In this manner, a source of neuronal apoptosis inhibitor protein is provided. Given the sequence of NAIP and the probes and primers therein, deletions in the sequence may also be detected, for instance, in the disorder Spinal Muscular Atrophy.

25 *NAIP Structure*

The NAIP gene contains 17 exons comprising at least 5.5 kb and spans an estimated 80 kb of genomic DNA. The NAIP coding region spans 3698 nucleotides resulting in a predicted gene product of 1233 amino acids. NAIP contains two potential transmembrane regions and an intracellular inhibitor of apoptosis domain immediately contiguous with a GTP binding site. Searches of the protein domain programs generated the following results:

- (i) residues 9-91: an N terminal domain with no recognizable motifs.
- (ii) residues 94-118: hydrophobic potential membrane spanning domain.

(iii) residues 169-485: a domain which shows homology with apoptosis inhibitors and is immediately before the next hydrophobic domain, GTP/ATP binding site.

(iv) residues 486-504: a hydrophobic potential membrane spanning domain.

(v) residues 505-1005: possible receptor domain containing 4 N-linked glycosylation sites and a lipoprotein binding domain

10 *Neuronal Apoptosis Inhibitor* *Protein Gene Mutational Analysis*

A cDNA20.3 probe was found by using the entire PAC 125D9 as a probe to screen cDNA libraries. Probing of genomic southern blots with cDNA probe 20.3 revealed the absence of a 9 kb *EcoRI* band in a Type III consanguineous family. This information mapped the NAIP gene deletions to exons 5 and 6. Thus the deletion covers the exon containing the rare *NotI* restriction site and the exon immediately downstream. Primers in and around these exons were constructed revealing the absence of amplification from 3 Type I and 3 Type III SMA individuals. Genomic DNA was isolated from PAC and cosmid subclones in and around exons 4 and 5 and sequenced in an effort to generate primers which would amplify the junction fragment generated by the causative deletions as depicted. A junction fragment was detected in the Type III individual. A similar product was observed in two other French Canadians with no history of consanguinity. The 3 Type I and 3 Type III SMA individual's chromosomes had identical CATT/CMS haplotypes strongly suggesting that this is a common mild SMA mutation and comparatively frequent in the French Canadian population. Cosegregation of this pattern was demonstrated. We have conducted analysis of 110 parents of SMA individuals and have failed to find a similar product. Sequencing of the genomic DNA in this region revealed an approximately 10 kb deletion resulting in an

in frame deletion. This deletion spans intron regions and exons 5 and 6. Southern blot analysis of two generation SMA families was performed. A cDNA probe encompassing the first eight exons was performed on
5 *EcoRI*-digested DNA from peripheral blood leukocytes. SMA affected members show an absence of hybridization to a 10 kb *EcoRI* band which was shown to contain exons 5 and 6 (Figure 9).

Initial isolation of the NAIP transcript was
10 achieved by probing a human fetal brain cDNA library with the entire 28 kb genomic DNA insert of cosmid 250B6 that contains one of five CATT subloci present in the cosmid library. This resulted in the detection of a 2.2 kb transcript that ultimately proved to be exon 14 of the
15 NAIP gene. Further probing of fetal brain libraries with the contiguous cosmid inserts (cosmid 40G1), as well as single copy subclones isolated from such cosmids identified a number of transcripts including the L7 transcript that ultimately proved to contain exon 13 of
20 the NAIP locus. No coding region was detected for L7, probably due to the fact that a substantial proportion of the clone contained unprocessed heteronuclear RNA, obscuring its true nature.

At this stage, the completed genetic and linkage
25 disequilibrium analyses and construction of the PAC contiguous array identified PAC 125D9 as having a good probability of containing the SMA locus. Four PAC 125D9 genomic libraries were constructed by performing complete and partial (average insert size 5 kb) *Sau3AI*,
30 *BamHI* and *BamHI/NotI* digests on the PAC insert and cloning the restricted products into plasmid vector. High through put genomic sequencing was conducted on both termini of 200 clones from the 5 kb insert partial *Sau3AI* digestion library in the manner of (Chen et al., 1993),
35 permitting the construction of contiguous and overlapping genomic clones covering most of PAC 125D9 (data not shown). This has proven instrumental in elucidating the

WO 96/12016

35

gene structure of the NAIP locus. PAC 125D9 is divided into 24 kb centromeric and 130 kb telomeric fragments by NotI digestion, bisecting exon 6 of the NAIP gene at the beginning of the first potential transmembrane domain mapping upstream of the inhibitor of apoptosis homologous domains (Figure 11 and Table 4). The NotI PAC fragments were isolated by preparative PFGE and used separately to probe human fetal brain cDNA libraries. Physical mapping and sequencing of the NotI site region was also undertaken to assay for the presence of a CpG island, an approach that rapidly detected coding sequence. The PAC was also used as a template in an exon trapping system (Church et al., 1994) resulting in the identification of the NAIP gene exons 5, 15 12, 16 and 17.

This multi-pronged approach resulted in the identification of cDNA clones spanning the NAIP gene (Figure 10). Overlapping clones were identified and chimerism of cDNA clones was excluded on a number of occasions by the detection of co-linearity of the cDNA clone termini with sequence from clones of the PAC 125D9 partial Sau3AI digestion genomic library. At this time, sequence analysis revealed the similarity between the protein sequence encoded by the NAIP gene exons 7 through 13 with two baculoviral inhibitor of apoptosis proteins (IAPs). Shortly thereafter, probing of Southern blots containing DNA from consanguineous SMA families with cDNA probes revealed deleted bands.

Both IAPs contain in their amino terminus an 80 amino acid EIR (baculovirus IAP repeat) motif that, after an intervening sequence of approximately 30 residues, is duplicated with 33% identity (Clem and Miller, 1993). The same phenomenon is observed in NAIP; amino acids 185-250 encoded by exons 6, 7 and 8 are 35% homologous to amino acids 300-370 encoded in exon 10, 11 and 12. The greatest stretch of homology is observed over a 53 amino acid region with 29 identical amino acids.

In addition to the NH2 terminal IAP domain, there exists cysteine and histidine rich zinc finger-like motifs in the carboxy terminus of both CpIAP and OpIAP. These motifs, which are proposed to interact with DNA (Birnbaum et al., 1994), are not seen in NAIP (Table 4). NAIP contains two potential transmembrane regions that bracket an inhibitor of apoptosis domain and a contiguous GTP binding site. Additional searches of protein domain programs generated the following more specific results than the aforementioned protein domain evaluation.

1. Residues 1-91: an N terminal domain with no recognizable motifs;
2. Residues 92-110: a hydrophobic domain predicted by the MEMSAT program (Jones et al., 1994) to be a membrane spanning domain;
3. Residues 163-477: a domain that shows homology with baculoviral inhibitors of apoptosis proteins followed by, and immediately upstream of the next hydrophobic domain, a GTP/ATP binding site;
4. Residues 479-496: hydrophobic domain predicted by MEMSAT to be a membrane spanning domain;
5. Residues 497-1232: a possible receptor domain containing four N-linked glycosylation sites and a procaryotic lipid attachment site.

We know of at least three exons that comprise 400 bp of 5' untranslated region (5'UTR); it is possible that more exist. A striking feature of this region is the presence of a perfect duplication of a 90 bp region in the 5' UTR before exon 2 and in the region bridging exons 2 and 3 (Table 4). In addition, the 3' untranslated

region comprising exon 17 has been found to contain a 550 bp interval that has potential coding region detected by the GRAIL program with high homology ($P=1.1e-37$) to the chicken integral membrane protein, occludin (Furuse et al., 1993). There exists, the possibility that this represents a chimeric transcript. Occludin homologous sequence has been detected in four different cDNA clones and two isoforms of the gene. The possibility of the occludin sequence representing a coding exon of the NAIP gene with the putative 3' UTR actually being heteronuclear RNA is also unlikely given the consistency with which the 3' UTR is observed and the presence of in frame translational stop codons mapping upstream of the region of occludin homology. Preliminary RT-PCR analysis indicates that the occludin tract is transcribed.

Tissue Expression

Hybridization of a Northern blot containing adult tissue mRNA with an exon 14 probe detected bands only in adult liver (approximately 6 and 7 kb bands) and placenta (7 kb, Figure 6). Although the level of expression in adult CNS is not sufficient to result in visible bands on Northern analysis, successful reverse transcriptase-PCR (RT-PCR) amplification of the NAIP transcript using spinal cord, fibroblast and lymphoblast RNA suggests transcriptional activity in these tissues.

Detection of Truncated and Internally Deleted Versions of the NAIP gene

In the analysis of the PAC contig, the clones 238D12 and 30B2 were noted to show significant sequence similarity with 125D9 but not to contain the NotI site in PAC 125D9 that is located in NAIP exon 6. This indicated the possibility of duplicated copies of the NAIP gene and so further analysis by hybridization of Southern blots containing PAC DNA with NAIP exon probes and PCR STS content assessment was undertaken. In this manner, two

aberrant versions of the NAIP locus were detected, one with exons 2 to 7 deleted (PAC 238D12), and another with exons 6, 7 and 12 to 15 deleted (PACs 30B2 and 250I7). The presence of identical sized bands in both genomic and PAC DNA on Southern blot analysis as well as PCR results outlined below obviate the possibility that the deletions represent in vitro PAC artifacts rather than the in vivo situation. Thus, genomic DNA Southern blots hybridized with NAIP exon probes revealed more bands than would be expected with a single intact copy of the NAIP gene. For example, probing of blots containing BamHI restricted genomic DNA with NAIP exons 3-11 should lead to a single band comprised of equal sized contiguous 14.5 kb BamHI fragments in the intact NAIP locus (Figure 11). Instead, two additional bands are seen at 9.4 and 23 kb (Figure 14), fragments that are seen in PACs 238D12 and 30B2/250I7 respectively. The 9.4 fragment BamHI has been subcloned from a cosmid and found to contain exons 8-11 with a deletion incorporating exons 2 to 7 occurring just upstream of the 8th exon (Figure 11). The 23 kb band is generated by a 6 kb deletion removing a BamHI site leading to the replacement of the two contiguous 14.5 kb BamHI fragments with a 23 BamHI fragment containing exons 2 to 5 and 8 to 11 and lacking exons 5 and 6 as depicted in Figure 11. The left side of this deletion was mapped by the fact that amplification with primers 1933 and 1926 generated a product whereas PCR with 1933 and 1923 did not (data not shown). PCR employing primers 1927 and 1933, constructed to amplify a 4.2 kb junction fragment spanning the 6 kb deletion (Figure 11), generated the appropriate product as shown by size and sequencing in both genomic DNA and PACs 30B2/250I7. The variable dosage of both the 9.4 and 23 kb bands seen in genomic DNA from different individuals indicates that the two partially deleted versions of the NAIP gene are present in multiple and polymorphic number in the general population.

A further level of complexity was detected with the identification of clones from a non-SMA human fetal brain cDNA library deleted for exons 11 and 12 (Scheme #1), some of which also had exons 15 and 16 (Scheme #1) absent (Figure 10). The fact that these deletions result in frame shifts and premature protein truncation indicates that they are, rather than normal splicing variants, more likely the result of transcription of the deleted and truncated version of NAIP gene that are present in the general population (Figure 11). In all, a profile of a region containing a variable number of copies of internally deleted and truncated versions of the NAIP locus, some of which are transcribed, has emerged from our analysis.

Probing of blots containing DNA from the somatic cell hybrid HHW 1064 (Gilliam et al., 1989) with NAIP exonic probes indicates that all forms of the NAIP gene are confined to the 30 Mb deleted region of 5q11-13.3 contained in the derivative chromosome 5 of this cell line. This finding has been confirmed by FISH probings with NAIP exon 13 probe (unpublished data).

NAIP Gene Mutational Analysis

Probing of genomic Southern blots with PCR amplified NAIP exons 3 to 10 revealed the absence of a 4.8 kb EcoRI/BamHI fragment containing exons 5 and 6 in the four affected individuals of consanguineous Type III SMA family 24561 (Figure 11 and 14). The same probing of BamHI digested DNA from this family revealed the absence of a 14.5 kb band also in keeping with a loss of exons 5 and 6 as outlined above (Figure 11 and 14). Similar results were observed in two other French Canadian SMA families that were also believed consanguineous.

In order to confirm the proposed deletion of exons 5 and 6, primers homologous to these exons were made (primers 1893, 1864, 1863, 1910 and 1887 identified by arrow in Figure 11. Results of a representative PCR

amplification of DNA from the family 24561 and a second Type III SMA consanguineous family using exon 5 specific primers (primer 1864 and 1863) along with a simultaneous reaction of an exon 13 sequence included to rule out a failure of the PCR are shown in Figure 15. Absence of amplification of exon 5 can be seen to cosegregate with the SMA phenotype.

In order to determine if the exon 5 and 6 NAIP gene deletion was an SMA mutation, Southern blot analysis was conducted. An 800 bp EcoRV single copy probe that mapped immediately to the 3' side of the 6 kb exon 5 and 6 deletion was employed (Figure 11). Hybridization of this marker to EcoRI Southern blots detected both a 9.4 kb EcoRI fragment containing exons 5 and 6 from the intact NAIP locus as well as a 3 kb EcoRI band from the exon 5 and 6 deleted copy of the NAIP gene. Analysis was conducted on EcoRI Southern blots containing DNA from over 900 unrelated members of myotonic dystrophy, ADPKD and cystic fibrosis families obtained from our DNA diagnostic laboratory. The 9.4 kb band was seen in all individuals in keeping with the presence of at least one copy of exons 5 and 6 in each of the approximately 900 individuals tested. In addition, the 3 kb band was observed in every individual reflecting a virtually complete dispersion of some form of the exon 5 through 6 deleted NAIP gene in the general population. Moreover, the variable band dosage observed for the 3 kb band suggested that the number of copies of the exon 5-6 deleted NAIP gene is polymorphic possibly ranging as high as 4 or 5 copies per genome.

PCR analysis was then extended to 110 SMA families, employing exon 5 and 6 primers. Seventeen of 38 (45%) Type I SMA individuals and 13 of 72 (18%) Type II and III SMA individuals were homozygously deleted for these exons. Assuming random assortment of chromosomes and therefore taking the square of the observed frequency of homozygous exon 5 through 6 deleted individuals yields

estimated frequencies for exon 5 through 6 deleted chromosomes of 67% in Type I SMA and 42% in Type II/III SMA. PCR analysis was next conducted on 168 parents of SMA children revealed failure of amplification suggesting homozygous deletion of exon 5 and 6 in three individuals. This finding was confirmed by Southern analysis in the two cases with sufficient DNA for this assay. The two individuals, aged 28 and 35 and both parents of Type I SMA children, when interviewed by telephone described themselves to be physically well, reporting no symptoms suggestive of SMA. It was thus concluded that the deletion of NAIPs exons 5 through 6 in isolation, while possibly reflecting more severe deletions in individuals with SMA as outlined below, can be clinically innocuous associated either with an exceedingly mild SMA or even normal phenotype. Clinical assessment of these individuals is currently being undertaken.

Judging both by the cDNA clones detected from fetal brain libraries as well as the make-up of RT-PCR NAIP products (Figure 2), many and possibly all truncated copies of the NAIP gene appear to be transcribed. Given the apparently unaffected status of the three parents of individuals with SMA who do not have a copy of exons 4 and 5 in their genome we believe that the exon 5 through 6 deleted version of NAIP is also translated. In keeping with this model, removal of exons 5 and 6 results in an in-frame deletion that extends the longest NAIP open reading frame upstream to a start methionine in exon 3 at nucleotide 211 (Table 4).

Furthermore, the protein sequence encoded by the deleted exon 5 and 6 IAP motif is approximately 35% homologous to the IAP motif encoded in exons 10 and 11 possibly accounting for the absence of discernible phenotype in the three exon 5 through 6 deleted individuals. One possible model is that a single copy of exon 5 through 6 deleted NAIP on each chromosome results in the mild SMA phenotype, while individuals with greater

than 3 or 4 copies of the exon 4-5 deleted NAIP locus are clinically unaffected. The possibility that duplication of the SMA gene underlies the disease has recently been proposed by DiDonato et al. (1994).

5 RT-PCR amplification of RNA from SMA and non-SMA tissue. The results of RT-PCR amplification using RNA from both non-SMA and SMA individuals as template are shown in Figure 16.

We have established that at least some of the
10 internally deleted and truncated NAIP versions are transcribed. In order to distinguish between transcripts from the intact NAIP gene which would produce a functional protein from those that would not, an effort was made to RT-PCR amplify transcripts that were as large
15 as possible. Given the 2.2 kb size of exon 14, this was found to be one which encompassed exon 2 and the 5' end of exon 13. No product was detected at the level of ethidium bromide staining after first round PCR. Therefore, second round nested amplification was
20 undertaken as described in respect of the previous description of Figure 16.

A representative subset of RT-PCR experiments are shown in Figure 16. PCR of reverse transcribed product using RNA from non-SMA tissues as template and reverse
25 transcribing from exons 10 or 13 consistently amplified product of the expected size. In contrast, similar RT-PCR experiments on RNA from SMA tissue revealed no amplification in five cases in keeping with the marked down regulation or complete absence of the intact
30 transcript in such individuals (Figure 16A). The RNA obtained from the SMA tissues was no more than 12 hours post-mortem. As we have no difficulty in amplifying intact NAIP transcript from normal tissue which is 24 hr post mortem, we do not believe the difficulty in
35 amplification arises from RNA degradation. Furthermore, difficulty with amplification was seen for all SMA tissues which suggests against the possibility that NAIP

is transcribed solely in the motor neuron with depletion of this cell type in SMA resulting in RT-PCR failure in spinal cord tissue.

In the cases where amplification was observed, sequencing of RT-PCR products has revealed the following findings, as shown in figures 16A, 16B and 16C:

- (i) an in-frame deletion of codons 153 and 190 from the 3' end of exon 5 from sample a9.
- (ii) deletion of exon 6 resulting in a frame shift with a stop codon occurring 73 nucleotides into exon 7 in a product amplified by exon 5 primer 1864 and exon 13 primer 1974 from sample a2.
- (iii) an approximate 50 nucleotide insertion in a product amplified by exon 4 primer 1886 and exon 13 primer 1974 from sample a7.
- (iv) deletion of a glutamic acid codon number 158 in exon 5 in association with deletion of exon 11 and 12 in a product amplified by exon 5 primer 1864 and exon 13 primer 1974 from sample a3.
- (v) deletion of exons 11 and 12 introducing a frame shift and a stop codon 14 nucleotides into exon 13 in a product amplified by exon primer 9 primer 1844 and exon 13 primer 1974 in sample a2, a3, a9 and a11.

In all, employing PCR on material reverse transcribed from exon 13, we have observed successful amplification of the appropriate product from all 12 non-SMA tissues attempted and in only one of 12 SMA tissues. In the latter case, sample a12, amplification was from exons 13 to 4 only, whether the transcript also incorporates exons 2 to 3 or 14 to 17 is unknown. We believe that these data provide strong evidence for NAIP being the SMA gene.

Role of NAIP Protein

The discovery of a neuronal apoptosis inhibitor protein gene in the SMA region of chromosome 5 demonstrates that the SMA condition is a result of

deletions in the apoptosis inhibitor protein domains. The long time survival of motoneurons is dependent on the production of complete neuronal apoptosis inhibitor protein. The deletion of the apoptosis inhibitor protein domain compromises the protein activity. We have demonstrated that approximately 70% of all SMA affected individuals have deletions of exons 5 and 6 of chromosome 5.

The identified region of 5q13.1 contains a variable number of copies of intact and partially deleted forms of the NAIP gene. While we cannot rule out the presence of additional loci in 5q13.1 that when mutated contribute to the SMA phenotype, we believe that mutations of NAIP gene are necessary and possibly sufficient for the genesis of SMA. In contrast to most autosomal recessive diseases where causal mutations are usually detected in the single copy of a given gene, we propose that an SMA chromosome is characterized by a paucity or, for severe SMA mutations, an absence of both the intact NAIP gene as well as that version which has had exons 3 and 4 deleted. The genesis of such chromosomes may involve unequal crossovers leaving the chromosome depleted for these loci with the resulting absence of the NAIP gene product leading to SMA.

25

Diagnosis of SMA

The delineation of an SMA genotype in a given individual is complicated by the unusual amplification of the NAIP gene in the 5q13.1 region. Probing of Southern blots containing genomic DNA with NAIP exon probes invariably reveal bands resulting from copies of internally deleted and truncated versions of the NAIP gene. The presence of variable numbers of the different forms of the NAIP loci in the general population is therefore the norm and not diagnostic of an SMA mutation per se, complicating the mutational analysis of the NAIP gene. If the detection of genomic DNA containing altered

NAIP loci affords no proof of an SMA chromosome then, by default, the search must be for the absence of the normal NAIP gene. However, we have detected rare individuals with no copies of exons 3-4 in their genome who are

5 clinically unaffected, an observation that is in keeping with what we know of NAIP gene structure. Consequently, the identification of an SMA chromosome is contingent on the absence of both the intact as well as the exons 3-4 only deleted forms of NAIP. Assaying for their absence

10 is complicated by the presence of segments of normal NAIP gene in each of the other, more extensively deleted, forms of the NAIP locus. One can see, for example, that if a given SMA individual had in their genome only the deleted versions of NAIP found on PACs 238D12 and 30B2,

15 that is exons 1-6 deleted and exons 5,6 and 11-14 deleted, respectively (Figures 10 and 11) in their genome, they would appear by PCR and Southern analysis to have the exons 5-6 only deleted version of NAIP, and therefore to have non-SMA chromosomes. We believe that

20 many and perhaps most of the numerous exon 5-6 deleted SMA individuals we have observed actually have chromosomes with such a configuration, containing neither the intact NAIP loci nor the exons 5-6 only deleted version but rather, some other combination of more

25 severely truncated/deleted versions of the locus with resultant absence of intact NAIP translation. Support for this interpretation comes from our inability to amplify normal NAIP transcripts employing RT-PCR on RNA from Type I SMA tissue.

30 In all, the evidence in support of mutations in or the absence of the NAIP gene causing SMA includes the following:

(i) The strong possibility that the NAIP, given its homology with baculoviral IAPs, functions as an inhibitor

35 of apoptosis. This characteristic is wholly compatible with the pathology of SMA. It is noteworthy that mutations in a regulator of apoptosis have been

previously suggested as a speculative cause of SMA (Oppenheim 1991, Sarnat, 1992).

- (ii) The mapping of the NAIP locus within the recombination defined critical SMA interval and the fact that the three polymorphic markers that have been shown to be in strong linkage disequilibrium with type I SMA; CATT-40GI (McLean et al., 1994), C272 (Melki et al., 1994) and AG-1 (DiDonato et al., 1994) all map to PAC 125D9 and are present on NAIP introns (Figure 9C).
- (iii) The nature of linkage disequilibrium observed between the type 1 SMA phenotype and the 5q13.1 markers. We have shown that the CATT-40GI CTR sublocus which is frequently duplicated on non-SMA chromosomes (Roy et al., 1994), is deleted in 80% of type 1 SMA chromosomes compared with 45% of non-SMA chromosomes (McLean et al., 1994). This finding is in keeping with a depletion of the number of NAIP genes on SMA chromosomes. In a similar fashion, Melki et al., 1994, have observed "a heterozygote deficiency" consisting of a reduced number of bands for the C272 CTR in Type I SMA, reflecting, they propose, chromosomal deletions. DiDonato et al., (1994) have also seen a striking reduction in the number of AG1 CTR sub-loci in Type I SMA individuals when compared with non-SMA individuals. We believe that the observation by three groups of the depletion of these intraNAIP markers on Type I SMA chromosomes fits well with the proposed model of a lack or absence of both the intact and exon 5-6 deleted form of the NAIP gene underlying the disease.
- (iv) The markedly increased frequency of NAIP exon 5-6 deletions observed in SMA chromosomes (approximately 67% of type 1 SMA chromosomes and 42% of type 2/3 SMA chromosomes) compared with that detected for non-SMA chromosomes (2-3%). As outlined above, we believe that this phenomenon reflects the rarity or absence of both the intact NAIP gene as well as the NAIP version with only exons 5 through 6 deleted in the SMA chromosomes, leaving only the more significantly internally deleted

and truncated forms of the NAIP gene present.

(v) Our consistent inability to RT-PCR amplify appropriate size transcripts from RNA obtained from 11 of 12 SMA individuals despite success with 12 of 12 RNAs from non-SMA individuals. Furthermore, sequencing of those RT-PCR products that could be obtained from type 1 SMA material revealed a variety of mutations and deletions.

(vi) The presence of a variable number of copies of truncated and internally deleted versions of the NAIP gene is similar to the situation reported in the autosomal dominant polycystic kidney disease gene (ADPKD, European Polycystic Kidney Disease Consortium, 1994). In this case portions of unprocessed pseudogenes corresponding to the causative gene were found to map elsewhere on chromosome 16p. The key difference, is that with the NAIP locus the mutated form of the gene is amplified.

In this regard the NAIP region of 5q13.1 has more similarity to the area of chromosome 6 containing CYP21, the gene that encodes steroid 21-hydroxylase (Wedell and Luthman, 1993). CYP21, which when mutated causes an autosomal recessive 21-hydroxylase deficiency, has been observed in 0-3 copies in individuals. There also exists in the region a variable number of inactive pseudogene copies of CYP21 known collectively as CYP21P. The majority of the CYP21 mutations that have been observed in 21-hydroxylase deficiency can also be found in some form of CYP21P and it is thought that the pseudogenes act as a source of the mutations observed in CYP21. The truncated and internally deleted NAIP genes are analogous to CYP21P only instead of the gene conversion postulated for CYP21/CYP21P it is possible that unequal crossing over results in chromosomes deleted for forms of the NAIP gene that encode functional protein. The existence of a polymorphic number of mutated NAIP genes on 5q13.1 is a credible mechanism for generation of SMA chromosomes in

this fashion.

Baculoviral IAPs

NAIP shows significant homology with the two
5 baculoviral gene products, CpIAP and OpIAP, that are
capable of inhibiting insect cell apoptosis (Table 4).
Insect cell apoptosis following baculoviral infection has
been well documented and is postulated to be a defence
mechanism. Premature death of infected insect cells
10 result in an attenuation of viral replication (Clem and
Miller, 1994a). CpIAP and OpIAP are thought to represent
baculoviral responses to this apoptotic mechanism. Both
act independently of other viral proteins to inhibit host
insect cell apoptosis, thereby permitting increased viral
15 proliferation (Clem and Miller, 1994a, 1994b). They are
known to be strongly similar only to each other; until
now no sequence similarities with cross phyla proteins
have been reported. Their mode of action is unknown,
although some interaction with DNA has been postulated.

20 The role and cellular localization of NAIP has not
yet been established. However, we believe that the
significant sequence similarity between NAIP and the
baculoviral IAPs, especially over such a considerable
phylogenetic distance, combined with the previously
25 postulated role of inappropriate apoptosis in the
pathogenesis of SMA make it likely that NAIP serves as an
apoptosis inhibitor in the motor neuron. Transfection
assays employing NAIP both in insect and mammalian
neuronal cells will help in this regard.

30 One possibility is that specific ligand binding of
the carboxy terminus of the NAIP activates the GTP
binding site which in turn activates the IAP domain. The
survival of a motor neuron might, therefore, be dependent
on the presence of the ligand(s): should the
35 concentration drop below a critical threshold, the IAP
domains cease to function with ensuing cell death. This
represents a possible mechanism for the natural winnowing

WO 96/12016

of motor neurons observed in embryogenesis. The source of the ligand might be postulated to be either muscle cells or Schwann cells. The embryogenesis of motor neurons might, therefore, be viewed as a competition between the cells with only those that make sufficient contacts to maintain the NAIP occupancy rate surviving. If, as postulated, NAIP does inhibit apoptosis, it is unclear whether NAIP is a constituent of a previously uncharacterized mammalian apoptotic pathway or a (presumably) upstream component of the pathway involving the human inhibitor of apoptosis, Bcl-2 (Vaux et al., 1988; Hockenberry et al., 1990; Garcia et al., 1992).

Assays employing apoptosis inhibition deficient baculoviral strains have revealed that Bcl-2 does not complement the deficiency in such assays (Clem and Miller, 1994b). If NAIP is a functional homolog of the intersection of a previously uncharacterized eucaryotic apoptotic pathway. One possibility is that NAIP represents an neurotrophic cytokine, ciliary neurotrophic factor (CNTF, Raff et al., 1993; Meakin and Shooter, 1993) or one of the downstream components of this pathway (Stahl et al., 1994). CNTF null mice show a pathologic picture that is similar to that of SMA with normal development of the neurons initially followed by their progressive apoptotic depletion (Masu et al., 1993). Moreover, although CNTF is alone among these agents in not depriving neurotrophins under the right conditions, it may result in apoptosis of cultured neurons, it is noteworthy that CNTF is alone among these agents in not having such apoptosis rescued by Bcl-2. This finding led the workers who made the observation to suggest the presence of a second eucaryotic apoptotic pathway (Allsopp et al., 1993). The existence of such distinct pathways may underlie the synergistic effect observed with the marked retardation of motor neuron loss in the wobbler mouse mutant following treatment with brain

derived neurotrophic factor (BDNF) and CNTF (Mitsumoto et al., 1994).

The role of the lipid attachment site in NAIP is unknown. Similar sites have been known to serve as
5 procaryotic protein leader sequences usually situated in the protein's amino terminus. We have detected the consensus pattern in 218 human sequences in the Swiss-Protein Database (release 28). These sequences are present in a variety of functional settings;
10 transmembrane regions, signal sequences, extracellular and cytoplasmic domains. One possibility is that the lipoprotein attachment site is extracellular and binds a constituent of the Schwann cell proteolipid in a manner that has been postulated for the apoptosis inhibiting
15 interaction of integrin with the extracellular matrix (Meredith et al., 1993; Frisch and Francis, 1994). Furthermore, the site may play a more active role in the hepatic form of the NAIP that we have observed on Northern blot analysis. It is noteworthy that serum
20 fatty acid abnormalities have been detected in children with SMA (Kelley and Sladky, 1986).

The identified region of 5q13.1 contains, in addition to the NAIP gene, a variable number of copies of internally deleted and truncated forms of the gene. We
25 believe that a lack or absence of both the intact NAIP gene and the NAIP locus with exons 5 and 6 deleted from a given individual's genome are likely to cause SMA. In this regard, the identification of NAIP has allowed us to develop accurate molecular based diagnoses of SMA as well
30 as directing the formulation of conventional and genetic therapies for these debilitating conditions.

Furthermore, the identification of genes showing homology with the NAIP locus and proteins that interact with NAIP may help in the continuing elucidation of apoptotic
35 mechanisms in mammalian cells.

EXAMPLES*Family Material*

Clinical diagnoses conducted as described in MacKenzie et al. (1993) with all patients fulfilling the
5 diagnostic criteria given therein. DNA was isolated from peripheral leukocytes as described (MacKenzie et al., 1993).

Genetic and Linkage Disequilibrium Analyses

10 Genotyping with microsattelite markers was as outlined in MacKenzie et al. (1993) and McLean et al. (1994). The following 5q13.1 loci were used as described: D5S112 (Brzustowicz et al., 1990), D5S351 (Hudson et al., 1992), D5S435 (Soares et al., 1993),
15 D5S557 (Francis et al., 1993), D5S629 and D5S637 (Clermont et al., 1994), D5S684 (Brahe et al., 1994), Y98T, Y97T, Y116T, Y122T and CMS (Kleyn et al., 1993), CATT (Burghes et al., 1994, McLean et al., 1994) and MAP1B (Lien et al., 1991).

20 Linkage disequilibrium analyses were conducted using parameters that can accommodate the multiple alleles seen with microsatellite repeats. Given the complexities inherent in disequilibrium analyses, a total of 4 different parameters for which multiple alleles may be
25 used were employed. These were D_{ij} , D_{ij}' and D' as defined in Hedrick (1987) as well as the chi square test. Two of these, D_{ij} and D_{ij}' have given the best a posteriori positional information in a previous study on myotonic dystrophy (Podolsky et al., 1994). The patient
30 and control population is as outlined in McLean et al. (1994).

Cosmid, YAC and PAC Arraying

Cosmid and YAC contig assembly was as outlined in
35 Roy et al. (1994). PACs were constructed as outlined in Ioannou et al. (1994). Using these procedures three PAC libraries have been constructed with a combined total of

175,000 clones and propagated as individual clones in microtiter dishes (Ioannou et al., unpublished results). Pools derived from the three libraries (designated LLNL PAC1, RPCI1 and RPCI2) were screened with 5q13.1 STS's.

- 5 Positive PACs were arranged into a contiguous and overlapping arrays by further analysis with additional STSs combined with probings of Southern blots containing PAC DNA by single copy genomic DNA and cDNA probes.

10 *DNA Manipulation and Analysis*

- Four genomic libraries containing PAC 125D9 insert were constructed by BamHI, BamHI/NotI, total and partial Sau3aI (selected for 5kb insert size) digestions of the PAC genomic DNA insert and subcloned into Bluescript
15 vector. Sequencing of approximately 400 bp of both termini of 200 five kb clones from the partial Sau3AI digestion library in the manner of Chen et al. (1993) was undertaken.

- Coding sequences from the PACs were isolated by the
20 exon amplification procedure as described by Church et al. (1994). PACs were digested with BamHI or BamHI and BglII and subcloned into pSPL3. Pooled clones of each PAC were transfected into COS-1 cells. After a 24h transfection total RNA was extracted. Exons were cloned
25 into pAMP10 (Gibco, BRL) and sequenced utilizing primer SD2 (GTG AAC TGC ACT GTG ACA AGC TGC).

- DNA sequencing was conducted on an ABI 373A automated DNA sequencer. Two commercial human fetal brain cDNA libraries in lambda gt (Stratagene) and lambda
30 ZAP (Clontech) were used for candidate transcript isolation. The Northern blot was commercially acquired (Clontech) and probing was performed using standard methodology.

- In general, primers used in the paper for PCR were
35 selected for T_m s of 60°C and can be used with the following conditions: 30 cycles of 94°C, 60s; 60°C, 60s; 72°C, 90s. PCR primer mappings are as referred to in the

figure legends and text. Primer sequences are as follows:

- 1258 ATg CTT ggA TCT CTA gAA Tgg - Sequence ID No. 3
- 5 1285 AgC AAA gAC ATg Tgg Cgg AA - Sequence ID No. 4
- 1343 CCA gCT CCT AgA gAA AgA Agg A - Sequence ID No. 5
- 1844 gAA CTA Cgg CTg gAC TCT TTT - Sequence ID No. 6
- 1863 CTC TCA gCC TgC TCT TCA gAT - Sequence ID No. 7
- 1864 AAA gCC TCT gAC gAg Agg ATC - Sequence ID No. 8
- 10 1884 CgA CTg CCT gTT CAT CTA CgA - Sequence ID No. 9
- 1886 TTT gTT CTC CAg CCA CAT ACT - Sequence ID No. 10
- 1887 CAT TTg gCA TgT TCC TTC CAA g - Sequence ID No. 11
- 1893 gTA gAT gAA TAC TgA TgT TTC ATA ATT - Sequence ID No. 12
- 15 1910 TgC CAC TgC CAg gCA ATC TAA - Sequence ID No. 13
- 1919 TAA ACA ggA CAC ggT ACA gTg - Sequence ID No. 14
- 1923 CAT gTT TTA AgT CTC ggT gCT CTg - Sequence ID No. 15
- 1926 TTA gCC AgA TgT gTT ggC ACA Tg - Sequence ID No. 16
- 1927 gAT TCT ATg TgA TAG gCA gCC A - Sequence ID No. 17
- 20 1933 gCC ACT gCT CCC gAT ggA TTA - Sequence ID No. 18
- 1974 gCT CTC AgC TgC TCA TTC AgA T - Sequence ID No. 19
- 1979 ACA AAg TTC ACC ACg gCT CTg - Sequence ID No. 20

RT-PCR

- 25 cDNA was synthesized in a 20 μ l reaction utilizing 7 μ g of total RNA. The RNA was denatured for 5 minutes at 95°C and cooled to 37°C. Reverse transcription was performed at 42°C for 1 hour after addition of 5 μ l 5X reverse transtriction buffer, 2 μ l 0.1 M DTT, 41 2.5 mM
- 30 dNTPs, 8 units RNasin, 25 ng cDNA primer (1285) and 400 units of MMLV (Gibco, BRL). 1 μ l of cDNA was utilized as template in subsequent 50 μ l PCR reactions. 1 μ l of this primary PCR was utilized as template for secondary PCR amplifications.

Sequence Analysis

Primary DNA sequence data was edited with the TED program (Gleeson and Hillier, 1991). As many of the partially sequenced 200 five kb clones from the partial
5 Sau3AI digestion library as possible were arranged into overlapping arrays using the XBAP Staden package (Dear and Staden, 1991). Sequence data was also assembled and analyzed using the GCG Sequence analysis (Genetics
10 computer group, 1991). Protein domain homologies were found by searching the Prosite Protein database (Bairoch and Bucher, 1993). The MEMSAT program was also used to search for transmembrane domain regions (Jones et al., 1994).

TABLE 1

The YACs isolated in this study, their size and library of origin are listed. NCE: National Centers of Excellence, Toronto, Ontario, Canada. ICRF: Imperial Cancer Research Fund, CEPH: Centre d'Etude du Polymorphisme Humaine.

YAC	SIZE	LIBRARY
12H1	560kb	NCE
12H4	270kb	NCE
24D6	750kb	NCE
27H5	630kb	NCE
33H10	1.3Mb	NCE
H0416	390kb	ICRF
E0320	440kb	ICRF
G1138	850kb	ICRF
A0848	350kb	ICRF
D06100	580kb	ICRF
D0981	450kb	ICRF
919C2	800kb	CEPH
755B12	1Mb	CEPH
754H5	500kb	CEPH

TABLE 2

PROBE	SOURCE/ REFERENCE	PROBE	SOURCE/ REFERENCE
YD33	STS developed from <i>Alu</i> -5'-trp PCR product of YAC D06100	Y13.1	STS developed from inter <i>Alu</i> -5' PCR product of YAC 12H1 (this study)
Y14.1	STS developed from <i>Alu</i> -3'-ura PCR product of YAC 12H4 (this study)	Y15.1	STS developed from <i>Alu</i> -5'-ura PCR product of YAC 12H4 (this study)
Y9.2	STS developed from inter- <i>Alu</i> -5' PCR product of YAC 27H5 (this study)	Y5.6	STS developed from inter- <i>Alu</i> -3' PCR product of YAC 24D6 (this study)
Y11.2	STS developed from <i>Alu</i> -3'-trp PCR product of YAC 33H10 (this study)	pZY8	subcloned 1.3 kb <i>Hind</i> III fragment from cosmid 250B6 (this study)
H7T733	<i>Alu</i> 33-T7 PCR product from cosmid 1H7 (this study)	p151.2	subcloned 1.2 kb inter- <i>Alu</i> PCR product of cosmid 15F8 (this study)
G10T333	<i>Alu</i> 33-T3 PCR product of cosmid IG10 (this study)	p402.1	subcloned 2.1 kb <i>Bam</i> HI/ <i>Hind</i> III fragment of cosmid 40GI (this study)
G3T733	<i>Alu</i> 33-T7 PCR product of cosmid IG3 (this study)	pL7	liver transcript isolated with subcloned 1.1 kb <i>Bam</i> HI/ <i>Sal</i> I fragment from 58G12 (this study)
p2281.8	subcloned 1.8 kb <i>Hind</i> III fragment of cosmid 228C8 (this study)	F933	inter- <i>Alu</i> PCR product of cosmid 1F9 (this study)
pGA1	fetal brain transcript isolated with cosmid 250B6	β -glucuronidase	(Oshima et al. 1987)
MAP1B	(Lien et al. 1991)	Y122T	(Kleyn et al., 1993)
D5S351	(Yaraghi et al., in press)	CMS-1	(Kleyn et al., 1993)

PROBE	SOURCE/ REFERENCE	PROBE	SOURCE/ REFERENCE
D5S557	(Francis et al., 1993)	Y98T	(Kleyn et al., 1993)
D5S112	(Brzustowicz et al., 1990)	Y97T	(Kleyn et al., 1993)
Y112U	(Kleyn et al., 1993)	Y88T	(Kleyn et al., 1993)
Y119T	(Kleyn et al., 1993)	Y116U	(Kleyn et al., 1993)
CATT-1	(Burghes et al., 1994; McLean et al., in press)	Y55U	(Kleyn et al., 1993)
D5S127	(Sherrington et al., 1991)	Y38T	(Kleyn et al., 1993)
D5S435	(Soares et al., 1993)	D5S125	(Hudson et al., 1992)
Y107U	(Kleyn et al., 1993)	Y97U	(Kleyn et al., 1993)
D5F149 (C212)	(Melki et al., 1994)	D5F151 (C171)	(Melki et al., 1994)
D5F150 (C272)	(Melki et al., 1994)	D5F153 (C161)	(Melki et al., 1994)
D5S637	(Clermont et al., 1994)	D5S629	(Clermont et al., 1994)

Table 3

The homology of the GAl component of neuronal apoptosis inhibitor protein gene compared for homology with the inhibitor apoptosis polypeptides of the viruses Cydia pomonella and Orgyia pseudotsugata.

	1	50
Cydia pomonella
Orgyia pseudots
cGAl-concensus	TRTVDKPQKM ATQQKASDER ISQFDHNLTP ELSALLGLDA VQLAKELEEE	
	51	100
Cydia pomonella
Orgyia pseudots
cGAl-concensus	EQKERAKMQK GYNSQMRSEA KRLKTFVITYE PYSSWIPQEM AAGGFYFTGV	
	101	150
Cydia pomonella
Orgyia pseudots
cGAl-concensus	KSGIQCFCCS LILFGAGLTR LPIDHKKRFH PDCGFLNKD VGNIAKYDIR	MS
	151	200
Cydia pomonellaM SCLR..LEEV RLNTFEKWP..VSFLSPETM AKNGFYTLGR	
Orgyia pseudots	SRAIGAPQEG ADMK..NKAA RLGTYNWP..VQFLEPSRM AASGFYTLGR	
cGAl-concensus	VKNLKSRLRG GKMYQESEA RLASFRNWPF YVQGISPCLV SEAGFVFTGK	
	201	250
Cydia pomonella	SDEVRCAPCK VEIMRWKEGE DPAADHKXWA PQCPFKGID VCGSI.....	
Orgyia pseudots	GDEVRCAPCK VEITNWVRGD DPETHKRWV PQCPFVRN.....	
cGAl-concensus	QDTVQCFSCG GCLGNWEEGD DPWKEHAKWF PKCEFLRSKK SSEEITQYIQ	
	251	300
Cydia pomonellaVTT NNIONTITHD TIIGPA....HPKYAHEAAR VKSFHNWPRC	
Orgyia pseudotsNA HDTPHDRAPP ARSAAA....HPQYATEAAR LRTFAEWPRG	
cGAl-concensus	SYKGFVDITG EHFVNSWVQR ELPASAYCN DSIFAYEELR LDSFKDWPRE	
	301	350
Cydia pomonella	MKQRPEQMAD AGFFYTGIGD NTKCFYCDGG LKDWEPEDVP WEQHVRFDR	
Orgyia pseudots	LKQRPEELAE AGFFYTGIGD KTRCFCCDGG LKDWEPDDAP WQOHARWYDR	
cGAl-concensus	SAVGVAALAK AGLFYTGIGD IVQCFSCGGC LEKWQEGDDP LDDHTRCFPN	
	351	400
Cydia pomonella	CAYVQLVKGR DYVQKVI...TEACVLPGEN TTVSTAAPVS EPIPETKIEK	
Orgyia pseudots	CEYVLLVKGR DFWQRM...TEACVVRDAD N.....EPHIER	
cGAl-concensus	CPFLQNMKSS AEVTPDLQSR GELCELLETT SESNLEDSIA VGPVPEMAQ	
	401	450
Cydia pomonellaEPQ VEDSKLCKIC YVEE.....CIV CFVPCGHVVA	
Orgyia pseudots	PAV.....EAE VADRLCKIC LGAE.....KTV CFVPCGHVVA	
cGAl-concensus	GEAQWFQEAQ NLNEQLRAAY TSASFHMSL LDISSDLATD HLLGCDLSIA	
	451	500
Cydia pomonella	CACALSVDK CPMCRKIVTS VLKVFYS.....	
Orgyia pseudots	CGKCAAGVTT CPVCRGQLDK AVRMYQV.....	
cGAl-concensus	SKHISKPVQE PLVLPEVFGN LNSVMVEGE AGSGKTVLLK KIAFLWASGC	

TABLE 3 (continued)

	501	550
cGA1-concensus	CPLLNRQVLV FYLSLSSTRP DEGLASIICD QLEKEGSVT EMCNRNIIQQ	
cGA1-concensus	LKNQVLFLLD DYKEICSIPQ VIGKLIQKNH LSRTCCLIAV RTNRARDIRR	
cGA1-concensus	YLETILEIQA FPFYNTVCIL RKLFSHNMTR LRKFMVYFGK NQSLQKIQT	
cGA1-concensus	PLFVAAICAH WFOYPPDPSF DDVAVFKSYM ERLSLRNKAT AEILKATVSS	
cGA1-concensus	CGELALKGFF SCCFEFNDDD LAEAGVDEDE DLTMCCLMSKF TAQRLRPFYR	
cGA1-concensus	FLSPAQQEFL AGMRLIELLD SDRQEHQDLG LYHLKQINSP MMTVSAYNNF	
cGA1-concensus	LNYVSSLPST KAGPKIVSHL LHLVDNKESL ENISENDDYL KHQPEISLQM	
cGA1-concensus	QLLRGLWQIC PQAYFSMVSE HLLVLALKTA YQSNVVAACS PFVLQFLQGR	
cGA1-concensus	TLTLGALNLQ YFFDHPESLS LLRSIHFSIR GNKTSPPRAHF SVLETCTFDKS	
cGA1-concensus	QVPTIDQDYA SAFEPMNEWE RNLAEKEDNV KSYMMDQORRA SPDLSGTGYWK	
cGA1-concensus	LSPKQYKIPC LEVDVNDIDV VGQDMLEILM TVFSASQRIE LHLNHSRGFI	
cGA1-concensus	ESIRPALELS KASVTKCSIS KLELSAAEQE LLLTLPSLES LEVSGTIQSQ	
cGA1-concensus	DQIFPNLDKF LCLKELSVDL EGNINVFSVI PEEFPNFHHM EKLLIQISAE	
cGA1-concensus	S	

TABLE 4

Complete cDNA sequence and predicted amino acid sequence of NAIP. Nucleotide residues G are in lower case "g" to clearly delineate the residue C. Exon boundaries are as marked. Arrows underline the perfect 90 nucleotide tandem repeat in the 5' UTR region. The deleted exons 5 and 6 are shaded in gray. The regions of intra-protein repeated amino acid homology in the IAP domain are underlined. Sequence comparison with baculovirus IAPs is shown, identical residues are shaded dark grey and similar residues are shaded light grey. CpIAP and OpIAP refer to the IAPs encoded by the baculoviruses, Cydia pomonella granulosis virus (CpGV) and orgyia pseudotsugata nuclear polyhydrosis virus (OpMNPV), respectively. The sequence comparison has been extended to the cysteine/histidine putative DNA interacting region of the baculoviral IAPs, no NAIP homology can be seen. The region showing significant similarity to chicken occludin is shown in grey in the 3' UTR.

61

[illegible]

SUBSTITUTE SHEET

TABLE 4 (continued)

62a

SUBSTITUTE SHEET (RULE 26)

[illegible]

ORF with homology occluding

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(1) APPLICANT:

(A) NAME: UNIVERSITY OF OTTAWA
(B) STREET: 550 Cumberland Street
(C) CITY: Ottawa,
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K1N 6N5
(G) TELEPHONE: 613-564-5804
(H) TELEFAX: 613-564-5952

(A) NAME: RESEARCH DEVELOPMENT CORPORATION OF JAPAN
(B) STREET: 4-1-8, Honcho, Kawaguchi-shi
(C) CITY: Saitama 332
(E) COUNTRY: Japan
(F) POSTAL CODE (ZIP): none.

(A) NAME: MacKENZIE, Alex E.
(B) STREET: 35 Rockcliffe Way
(C) CITY: Ottawa
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K1N 1A3

(A) NAME: KORNELUK, Robert G.
(B) STREET: 1901 Tweed Avenue
(C) CITY: Ottawa
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K1G 2L8

(A) NAME: MAHADEVAN, Mani S.
(B) STREET: 818 South Grammon Road, Apt. 4
(C) CITY: Madison
(D) STATE: Wisconsin
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 53719

(A) NAME: McLEAN, Michael
(B) STREET: 1 Halesmanor Crt.
(C) CITY: Guelph
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): N1G 4E1

(A) NAME: ROY, Natalie
(B) STREET: 6 McLeod Street,
(C) CITY: Ottawa
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): K2P 0Z5

(A) NAME: IKEDA, Joh-E.
(B) STREET: 31-1 Kamineguro 5-chome, Meguro-ku,
(C) CITY: Tokyo
(E) COUNTRY: Japan
(F) POSTAL CODE: 153

(ii) TITLE OF INVENTION: NEURONAL APOPTOSIS INHIBITOR PROTEIN,
GENE SEQUENCE AND MUTATIONS CAUSATIVE OF SPINAL MUSCULAR ATROPHY

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5502 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(viii) POSITION IN GENOME:

(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTCCGGCTGG ACGTTGCCCT GTGTACCTCT TCGACTGCCT GTTCATCTAC GACGAACCCC	60
GGGTATTGAC CCCAGACAAC AATGCCACTT CATATTGCAT GAAGACAAAA GGTCCCTGTGC	120
TCACCTGGGA CCCTTCTGGA CGTTGCCCTG TGTTCTCTCT CGCCTGCCTG TTCATCTACG	180
ACGAACCCCG GGTATTGACC CCAGACAACA ATGCCACTTC ATATTGGGGA CTTCGTCTGG	240
GATTCCAAGG TGCATTCAAT GCAAAGTTCC TTAAATATTT TCTCACTGCT TCCTACTAAA	300
GGACGGACAG AGCATTGTGT CTTCAGCCAC ATACTTTCCT TCCACTGGCC AGCATTCTCC	360
TCTATTAGAC TAGAACTGTG GATAAACCTC AGAAAATGGC CACCCAGCAG AAAGCCTCTG	420
ACGAGAGGAT CTCCAGTTT GATCACAATT TGCTGCCAGA GCTGTCTGCT CTTCTGGGCC	480
TAGATGCAGT TCAGTTGGCA AAGGAAC TAGAAGAGGA GCAGAAGGAG CGAGCAAAAA	540
TGCAGAAAGG CTACAACTCT CAAATGCGCA GTGAAGCAAA AAGGTTAAAG ACTTTTGTGA	600
CTTATGAGCC GTACAGCTCA TGGATACCAC AGGAGATGGC GGCCGCTGGG TTTTACTTCA	660
CTGGGGTAAA ATCTGGGATT CAGTGCTTCT GCTGTAGCCT AATCCTCTTT GGTGCCGGCC	720
TCACGAGACT CCCCATAGAA GACCACAAGA GTTTTCATCC AGATTGTGGG TTCCTTTTGA	780
ACAAGGATGT TGGTAACATT GCCAAGTACG ACATAAGGGT GAAGAATCTG AAGAGCAGGC	840
TGAGAGGAGG TAAATGAGG TACCAAGAAG AGGAGGCTAG ACTTGCATCC TTCAGGAAC	900
GGCCATTTTA TGTCCAAGGG ATATCCCCTT GTGTGCTCTC AGAGGCTGGC TTTGTCTTTA	960

CAGGTAAACA	GGACACGGTA	CAGTGTTTTT	CCTGTGGTGG	ATGTTTAGGA	AATTGGGAAG	1020
AAGGAGATGA	TCCTTGGAAG	GAACATGCCA	AATGGTTCCC	CAAATGTGAA	TTTCTTCGGA	1080
GTAAGAAATC	CTCAGAGGAA	ATTACCCAST	ATATTCAAAG	CTACAAGGGA	TTTGTTGACA	1140
TAACGGGAGA	ACATTTTGTG	AATTCCTGGG	TCCAGAGAGA	ATTACCTATG	GCATCAGCTT	1200
ATTGCAATGA	CAGCATCTTT	GCTTACGAAG	AACTACGGCT	GGACTCTTTT	AAGGACTGGC	1260
CCCGGGAATC	AGCTGTGGGA	GTTGCAGCAC	TGGCCAAAGC	AGGTCTTTTC	TACACAGGTA	1320
TAAAGGACAT	CGTCCAGTGC	TTTTCTGTG	GAGGGTGTTT	AGAGAAATGG	CAGGAAGGTG	1380
ATGACCCATT	AGACGATCAC	ACCAGATGTT	TTCCCAATTG	TCCATTTCTC	CAAAATATGA	1440
AGTCCTCTGC	GGAAGTGA	CCAGACCTTC	AGAGCCGTGG	TGAACTTTGT	GAATTACTGG	1500
AAACCACAAG	TGAAAGCAAT	CTTGAAGATT	CAATAGCAGT	TGGTCCTATA	GTGCCAGAAA	1560
TGGCACAGGG	TGAAGCCCAG	TGGTTTCAAG	AGGCAAAGAA	TCTGAATGAG	CAGCTGAGAG	1620
CAGCTTATAC	CAGCGCCAGT	TTCCGCCACA	TGTCTTTGCT	TGATATCTCT	TCCGATCTGG	1680
CCACGGACCA	CTTGCTGGGC	TGTGATCTGT	CTATTGCTTC	AAAACACATC	AGCAAACCTG	1740
TGCAAGAACC	TCTGGTGCTG	CCTGAGGTCT	TTGGCAACTT	GAATCTGTCT	ATGTGTGTGG	1800
AGGGTGAAGC	TGGAAGTGGG	AAGACGGTCC	TCCTGAAGAA	AATAGCTTTT	CTGTGGGCAT	1860
CTGGATGCTG	TCCCCTGTTA	AACAGGTTC	AGCTGGTTTT	CTACCTCTCC	CTTAGTTCCA	1920
CCAGACCAGA	CGAGGGGCTG	GCCAGTATCA	TCTGTGACCA	GCTCCTAGAG	AAAGAAGGAT	1980
CTGTTACTGA	AATGTGCATG	AGGAACATTA	TCCAGCAGTT	AAAGAATCAG	GTCTTATTCC	2040
TTTTAGATGA	CTACAAAGAA	ATATGTTCAA	TCCCTCAAGT	CATAGGAAAA	CTGATTCAAA	2100
AAAACCACTT	ATCCCGGACC	TGCCTATTGA	TTGCTGTCCG	TACAAACAGG	GCCAGGGACA	2160
TCCGCCGATA	CCTAGAGACC	ATTCTAGAGA	TCCAAGCATT	TCCCTTTTAT	AATACTGTCT	2220
GTATATTACG	GAAGCTCTTT	TCACATAATA	TGACTCGTCT	GCGAAAGTTT	ATGGTTTACT	2280
TTGGAAAGAA	CCAAAGTTTG	CAGAAGATAC	AGAAAACCTC	TCTCTTTGTG	GCGGCGATCT	2340
GTGCTCATTG	GTTTCAGTAT	CCTTTTGACC	CATCCTTTGA	TGATGTGGCT	GTTTTCAAGT	2400
CCTATATGGA	ACGCCTTTCC	TTAAGGAACA	AAGCGACAGC	TGAAATTCTC	AAAGCAACTG	2460
TGTCCTCTCG	TGGTGACCTG	GCCTTGAAG	GGTTTTTTTC	ATGTTGCTTT	GAGTTTAATG	2520
ATGATGATCT	CGCAGAAGCA	GGGGTTGATG	AAGATGAAGA	TCTAACCATG	TGCTTGATGA	2580
GCAAATTTAC	AGCCCAGAGA	CTAAGACCAT	TCTACCGGTT	TTTAAGTCCT	GCCTTCCAAG	2640
AATTTCTTGC	GGGGATGAGG	CTGATTGAAC	TCCTGGATTG	AGATAGGCAG	GAACATCAAG	2700
ATTTGGGACT	GTATCATTTG	AAACAAATCA	ACTCACCCAT	GATGACTGTA	AGCGCCTACA	2760
ACAAATTTTT	GAATATGTC	TCCAGCCTCC	CTTCAACAAA	AGCAGGGCCC	AAAATTGTGT	2820
CTCATTTGCT	CCATTTAGTG	GATAACAAAG	AGTCATTGGA	GAATATATCT	GAAAATGATG	2880
ACTACTTAAA	GCACCAGCCA	GAAATTTTAC	TGCAGATGCA	GTTACTTAGG	GGATTGTGGC	2940

AAATTTGTCC	ACAAGCTTAC	TTTTCAATGG	TTTCAGAACA	TTTACTGGTT	CTTGCCCTGA	3000
AAACTGCTTA	TCAAAGCAAC	ACTGTTGCTG	CGTGTTCTCC	ATTGTTTTTG	CAATTCCTTC	3060
AAGGGAGAAC	ACTGACTTTG	GGTGCGCTTA	ACTTACAGTA	CTTTTTCGAC	CACCCAGAAA	3120
GCTTGTCAAT	GTTGAGGAGC	ATCCACTTCT	CAATACGAGG	AAATAAGACA	TCACCCAGAG	3180
CACATTTTTTC	AGTTCTGGAA	ACATGTTTTG	ACAAATCACA	GGTGCCAACT	ATAGATCAGG	3240
ACTATGCTTC	TGCCTTTGAA	CCTATGAATG	AATGGGAGCG	AAATTTAGCT	GAAAAAGAGG	3300
ATAATGTAAA	GAGCTATATG	GATATGCAGC	GCAGGGCATC	ACCAGACCTT	AGTACTGGCT	3360
ATTGGAAACT	TTCTCCAAAG	CAGTACAAGA	TTCCCTGTCT	AGAAGTCGAT	GTGAATGATA	3420
TTGATGTTGT	AGGCCAGGAT	ATGCTTGAGA	TTCTAATGAC	AGTTTTCTCA	GCTTCACAGC	3480
GCATCGAACT	CCATTTAAAC	CACAGCAGAG	GCTTTATAGA	AAGCATCCGC	CCAGCTCTTG	3540
AGCTGTCTAA	GGCCTCTGTC	ACCAAGTGCT	CCATAAGCAA	GTTGGAACTC	AGCGCAGCCG	3600
AACAGGAACT	GCTTCTCACC	CTGCCTTCCC	TGGAATCTCT	TGAAGTCTCA	GGGACAATCC	3660
AGTCACAAGA	CCAAATCTTT	CCTAATCTGG	ATAAGTTCCT	GTGCCTGAAA	GAACTGTCTG	3720
TGGATCTGGA	GGGCAATATA	AATGTTTTTT	CAGTCATTCC	TGAAGAATTT	CCAAACTTCC	3780
ACCATATGGA	GAAATTATTG	ATCCAAATTT	CAGCTGAGTA	TGATCCTTCC	AAACTAGTTG	3840
CCAGTTTGCC	AAATTTTATT	TCTCTGAAGA	TATTAAATCT	TGAAGGCCAG	CAATTTCTCTG	3900
ATGAGGAAAC	ATCAGAAAAA	TTTGCCTACA	TTTtaggttc	TCTTAGTAAC	CTGGAAGAAT	3960
TGATCCTTCC	TACTGGGGAT	GGAATTTATC	GAGTGGCCAA	ACTGATCATC	CAGCAGTGTC	4020
AGCAGCTTCA	TTGTCTCCGA	GTCCTCTCAT	TTTTCAAGAC	TTTGAATGAT	GACAGCGTGG	4080
TGGAAATTGG	TTAAAAATGT	GTCTGCAGGC	ACACAGGACG	TGCCTTCACC	CCCATCTGAC	4140
TATGTGGAAA	GAGTTGACAG	TCCCATGGCA	TACTCTTCCA	ATGGCAAAGT	GAATGACAAG	4200
CGGTTTTATC	CAGAGTCTTC	CTATAAATCC	ACGCCGGTTC	CTGAAGTGGT	TCAGGAGCTT	4260
CCATTAACTT	CGCCTGTGGA	TGACTTCAGG	CAGCCTCGTT	ACAGCAGCGG	TGGTAACTTT	4320
GAGACACCTT	CAAAAAGAGC	ACCTGCAAAG	GGAAGAGCAG	GAAGGTCAAA	GAGAACAGAG	4380
CAAGATCACT	ATGAGACAGA	CTACACAAC	GGCGGCGAGT	CCTGTGATGA	GCTGGAGGAG	4440
GACTGGATCA	GGGAATATCC	ACCTATCACT	TCAGATCAAC	AAAGACAAC	GTACAAGAGG	4500
AATTTTGACA	CTGGCCTACA	GGAATACAAG	AGCTTACAAT	CAGAACTTGA	TGAGATCAAT	4560
AAAGAACTCT	CCCCTTTGGA	TAAAGAATTG	GATGACTATA	GAGAAGAAAG	TGAAGAGTAC	4620
ATGGCTGCTG	CTGATGAATA	CAATAGACTG	AAGCAAGTGA	AGGGATCTGC	AGATTACAAA	4680
AGTAAGAAGA	ATCATTGCAA	GCAGTTAAAC	AGCAAATTGT	CACACATCAA	GAAGATGGTT	4740
GGAGACTATG	ATAGACAGAA	AACATAGAAG	GCTGATGCCA	AGTTGTTTGA	GAAATTAAGT	4800
ATCTGACATC	TCTGCAATCT	TCTCAGAAGG	CAAATGACTT	TGGACCATAA	CCCCGGAAGC	4860
CAAACCTCTG	TGAGCATCAC	AGTTTTGGTT	GCTTTAATAT	CATCAGTATT	GAAGCATTTT	4920

CACTTTTTTC	CACATAAGGA	AACTGGGTTT	CTGCAATGAA	GTCTCTGAAG	TGAAACTGCT	5220
TGTTTCCTAG	CACACACTTT	TGGTTAAGTC	TGTTTTATGA	CTTCATTAAT	AATAAATTCC	5280
GGCATCATAC	AGCTACTCCT	CCCTACCGCC	ACCTCCACAG	ACACCACTCT	CCTGGTTCCA	5340
TCTCCTCTGC	TGCTTCTAGC	TCCCTGCTCT	GGCTTCAAGG	TGCGCAGGAC	CTGCTTCCTT	5400
GGTGATCCTC	TGTAGTCTCC	CACACCCAC	ATTATCTACA	AACTGATGAC	TCCTAATTTA	5460
CATCTCCAGC	TCAGACCTCT	CCATCAATCC	CAACGCATAC	AC		5502

SUBSTITUTE SHEET (RULE 26)

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1233 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Ala Thr Gln Gln Lys Ala Ser Asp Glu Arg Ile Ser Gln Phe Asp
 1           5           10
His Asn Leu Leu Pro Glu Leu Ser Ala Leu Leu Gly Leu Asp Ala Val
          20           25           30
Gln Leu Ala Lys Glu Leu Glu Glu Glu Gln Lys Glu Arg Ala Lys
          35           40           45
Met Gln Lys Gly Tyr Asn Ser Gln Met Arg Ser Glu Ala Lys Arg Leu
          50           55           60
Lys Thr Phe Val Thr Tyr Glu Pro Tyr Ser Ser Trp Ile Pro Gln Glu
          65           70           75           80
Met Ala Ala Ala Gly Phe Tyr Phe Thr Gly Val Lys Ser Gly Ile Gln
          85           90           95
Cys Phe Cys Cys Ser Leu Ile Leu Phe Gly Ala Gly Leu Thr Arg Leu
          100          105          110
Pro Ile Glu Asp His Lys Arg Phe His Pro Asp Cys Gly Phe Leu Leu
          115          120          125
Asn Lys Asp Val Gly Asn Ile Ala Lys Tyr Asp Ile Arg Val Lys Asn
          130          135          140
Leu Lys Ser Arg Leu Arg Gly Gly Lys Met Arg Tyr Gln Glu Glu Glu
          145          150          155          160
Ala Arg Leu Ala Ser Phe Arg Asn Trp Pro Phe Tyr Val Gln Gly Ile
          165          170          175
Ser Pro Cys Val Leu Ser Glu Ala Gly Phe Val Phe Thr Gly Lys Gln
          180          185          190
Asp Thr Val Gln Cys Phe Ser Cys Gly Gly Cys Leu Gly Asn Trp Glu
          195          200          205
Glu Gly Asp Asp Pro Trp Lys Glu His Ala Lys Trp Phe Pro Lys Cys
          210          215          220
Glu Phe Leu Arg Ser Lys Lys Ser Ser Glu Glu Ile Thr Gln Tyr Ile
          225          230          235          240
Gln Ser Tyr Lys Gly Phe Val Asp Ile Thr Gly Glu His Phe Val Asn
          245          250          255
Ser Trp Val Gln Arg Glu Leu Pro Met Ala Ser Ala Tyr Cys Asn Asp
          260          265          270
Ser Ile Phe Ala Tyr Glu Glu Leu Arg Leu Asp Ser Phe Lys Asp Trp
          275          280          285
Pro Arg Glu Ser Ala Val Gly Val Ala Ala Leu Ala Lys Ala Gly Leu
          290          295          300

```

RECTIFIED SHEET (RULE 91)
 ISA/EP

69

Phe Tyr Thr Gly Ile Lys Asp Ile Val Gln Cys Phe Ser Cys Gly Gly
 305 310 315 320
 Cys Leu Glu Lys Trp Gln Glu Gly Asp Asp Pro Leu Asp Asp His Thr
 325 330 335
 Arg Cys Phe Pro Asn Cys Pro Phe Leu Gln Asn Met Lys Ser Ser Ala
 340 345 350
 Glu Val Thr Pro Asp Leu Gln Ser Arg Gly Glu Leu Cys Glu Leu Leu
 355 360 365
 Glu Thr Thr Ser Glu Ser Asn Leu Glu Asp Ser Ile Ala Val Gly Pro
 370 375 380
 Ile Val Pro Glu Met Ala Gln Gly Glu Ala Gln Trp Phe Gln Glu Ala
 385 390 395 400
 Lys Asn Leu Asn Glu Gln Leu Arg Ala Ala Tyr Thr Ser Ala Ser Phe
 405 410 415
 Arg His Met Ser Leu Leu Asp Ile Ser Ser Asp Leu Ala Thr Asp His
 420 425 430
 Leu Leu Gly Cys Asp Leu Ser Ile Ala Ser Lys His Ile Ser Lys Pro
 435 440 445
 Val Gln Glu Pro Leu Val Leu Pro Glu Val Phe Gly Asn Leu Asn Ser
 450 455 460
 Val Met Cys Val Glu Gly Glu Ala Gly Ser Gly Lys Thr Val Leu Leu
 465 470 475 480
 Lys Lys Ile Ala Phe Leu Trp Ala Ser Gly Cys Cys Pro Leu Leu Asn
 485 490 495
 Arg Phe Gln Leu Val Phe Tyr Leu Ser Leu Ser Ser Thr Arg Pro Asp
 500 505 510
 Glu Gly Leu Ala Ser Ile Ile Cys Asp Gln Leu Leu Glu Lys Glu Gly
 515 520 525
 Ser Val Thr Glu Met Cys Met Arg Asn Ile Ile Gln Gln Leu Lys Asn
 530 535 540
 Gln Val Leu Phe Leu Leu Asp Asp Tyr Lys Glu Ile Cys Ser Ile Pro
 545 550 555 560
 Gln Val Ile Gly Lys Leu Ile Gln Lys Asn His Leu Ser Arg Thr Cys
 565 570 575
 Leu Leu Ile Ala Val Arg Thr Asn Arg Ala Arg Asp Ile Arg Arg Tyr
 580 585 590
 Leu Glu Thr Ile Leu Glu Ile Gln Ala Phe Pro Phe Tyr Asn Thr Val
 595 600 605
 Cys Ile Leu Arg Lys Leu Phe Ser His Asn Met Thr Arg Leu Arg Lys
 610 615 620
 Phe Met Val Tyr Phe Gly Lys Asn Gln Ser Leu Gln Lys Ile Gln Lys
 625 630 635 640
 Thr Pro Leu Phe Val Ala Ala Ile Cys Ala His Trp Phe Gln Tyr Pro
 645 650 655

70

Phe Asp Pro Ser Phe Asp Asp Val Ala Val Phe Lys Ser Tyr Met Glu
 660 665 670
 Arg Leu Ser Leu Arg Asn Lys Ala Thr Ala Glu Ile Leu Lys Ala Thr
 675 680 685
 Val Ser Ser Cys Gly Glu Leu Ala Leu Lys Gly Phe Phe Ser Cys Cys
 690 695 700
 Phe Glu Phe Asn Asp Asp Asp Leu Ala Glu Ala Gly Val Asp Glu Asp
 705 710 715 720
 Glu Asp Leu Thr Met Cys Leu Met Ser Lys Phe Thr Ala Gln Arg Leu
 725 730 735
 Arg Pro Phe Tyr Arg Phe Leu Ser Pro Ala Phe Gln Glu Phe Leu Ala
 740 745 750
 Gly Met Arg Leu Ile Glu Leu Leu Asp Ser Asp Arg Gln Glu His Gln
 755 760 765
 Asp Leu Gly Leu Tyr His Leu Lys Gln Ile Asn Ser Pro Met Met Thr
 770 775 780
 Val Ser Ala Tyr Asn Asn Phe Leu Asn Tyr Val Ser Ser Leu Pro Ser
 785 790 795 800
 Thr Lys Ala Gly Pro Lys Ile Val Ser His Leu Leu His Leu Val Asp
 805 810 815
 Asn Lys Glu Ser Leu Glu Asn Ile Ser Glu Asn Asp Asp Tyr Leu Lys
 820 825 830
 His Gln Pro Glu Ile Ser Leu Gln Met Gln Leu Leu Arg Gly Leu Trp
 835 840 845
 Gln Ile Cys Pro Gln Ala Tyr Phe Ser Met Val Ser Glu His Leu Leu
 850 855 860
 Val Leu Ala Leu Lys Thr Ala Tyr Gln Ser Asn Thr Val Ala Ala Cys
 865 870 875 880
 Ser Pro Phe Val Leu Gln Phe Leu Gln Gly Arg Thr Leu Thr Leu Gly
 885 890 895
 Ala Leu Asn Leu Gln Tyr Phe Phe Asp His Pro Glu Ser Leu Ser Leu
 900 905 910
 Leu Arg Ser Ile His Phe Ser Ile Arg Gly Asn Lys Thr Ser Pro Arg
 915 920 925
 Ala His Phe Ser Val Leu Glu Thr Cys Phe Asp Lys Ser Gln Val Pro
 930 935 940
 Thr Ile Asp Gln Asp Tyr Ala Ser Ala Phe Glu Pro Met Asn Glu Trp
 945 950 955 960
 Glu Arg Asn Leu Ala Glu Lys Glu Asp Asn Val Lys Ser Tyr Met Asp
 965 970 975
 Met Gln Arg Arg Ala Ser Pro Asp Leu Ser Thr Gly Tyr Trp Lys Leu
 980 985 990
 Ser Pro Lys Gln Tyr Lys Ile Pro Cys Leu Glu Val Asp Val Asn Asp
 995 1000 1005

70 a

Ile Asp Val Val Gly Gln Asp Met Leu Glu Ile Leu Met Thr Val Phe
 1010 1015 1020
 Ser Ala Ser Gln Arg Ile Glu Leu His Leu Asn His Ser Arg Gly Phe
 1025 1030 1035 1040
 Ile Glu Ser Ile Arg Pro Ala Leu Glu Leu Ser Lys Ala Ser Val Thr
 1045 1050 1055
 Lys Cys Ser Ile Ser Lys Leu Glu Leu Ser Ala Ala Glu Gln Glu Leu
 1060 1065 1070
 Leu Leu Thr Leu Pro Ser Leu Glu Ser Leu Glu Val Ser Gly Thr Ile
 1075 1080 1085
 Gln Ser Gln Asp Gln Ile Phe Pro Asn Leu Asp Lys Phe Leu Cys Leu
 1090 1095 1100
 Lys Glu Leu Ser Val Asp Leu Glu Gly Asn Ile Asn Val Phe Ser Val
 1105 1110 1115 1120
 Ile Pro Glu Glu Phe Pro Asn Phe His His Met Glu Lys Leu Leu Ile
 1125 1130 1135
 Gln Ile Ser Ala Glu Tyr Asp Pro Ser Lys Leu Val Ala Ser Leu Pro
 1140 1145 1150
 Asn Phe Ile Ser Leu Lys Ile Leu Asn Leu Glu Gly Gln Gln Phe Pro
 1155 1160 1165
 Asp Glu Glu Thr Ser Glu Lys Phe Ala Tyr Ile Leu Gly Ser Leu Ser
 1170 1175 1180
 Asn Leu Glu Glu Leu Ile Leu Pro Thr Gly Asp Gly Ile Tyr Arg Val
 1185 1190 1195 1200
 Ala Lys Leu Ile Ile Gln Gln Cys Gln Gln Leu His Cys Leu Arg Val
 1205 1210 1215
 Leu Ser Phe Phe Lys Thr Leu Asn Asp Asp Ser Val Val Glu Ile Gly
 1220 1225 1230

*

RECTIFIED SHEET (RULE 91)
ISA/EP

71

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGCTTGGAT CTCTAGAATG G 21

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCAAAGACA TGTGGCGGAA 20

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCAGCTCCTA GAGAAAGAAG GA 22

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAACTACGGC TGGACTCTTT T 21

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTCTCAGCCT GCTCTTCAGA T 21

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAAGCCTCTG ACGAGAGGAT C 21

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CGACTGCCTG TTCATCTACG A 21

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTTGTCTCCTCC AGCCACATAC T 21

73

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CATTGGCAT GTTCCTTCCA AG 22

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTAGATGAAT ACTGATGTTT CATAATT 27

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGCCACTGCC AGGCAATCTA A 21

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TAAACAGGAC ACGGTACAGT G 21

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CATGTTTAA GTCTCGGTGC TCTG 24

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTAGCCAGAT GTGTTGGCAC ATG 23

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GATTCTATGT GATAGGCAGC CA 22

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

75

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCCACTGCTC CCGATGGATT A 21

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCTCTCAGCT GCTCATTCAG AT 22

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ACAAAGTTCA CCACGGCTCT G 21

REFERENCES

1. Albertson, H.M., Abderrahim, H., Cann, H.M., Dausset, J., le Paslier, D. and Cohen, D. (1990). Construction and characterization of a yeast artificial chromosome library containing seven haploid genome equivalents. *Proc. Nat Acad. Sci. USA.* 87: 4256-4260.
2. Allsopp, T.E. Wyatt, S., Paterson, H.F., and Davies, A.M. (1993). The Proto-Oncogene bcl-2 Can Selectively Rescue Neutrophic Factor-Dependent Neurons from Apoptosis. *Cell* 3073, 295-307.
3. Bairoch, A., and Bucher, P., (1994) PROSITE: Recent Developments. *Nucl. Acids Res.* 22:3583-3589.
4. Birnbaum, M.J., Clem, R.J., and Miller, L.K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys-His sequence Motifs. *J. Virol.* 68, 2521-2528.
5. Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids. Res.* 7: 1513-1523.
6. Boultonwood, J., Fidler, C., Lewis, S., Kelly, S., Sheridan, H., Littlewood, T.J., Buckle, V.J. and Wainscoat, J.S. (1994). Molecular mapping of uncharacteristically small 5q deletions in two patients with the 5q- syndrome: delineation of the critical region on 5q and identification of a 5q- breakpoint. *Genomics* 19: 425-432,
7. Burghes, A.H.M., Ingraham, S.E., McLean, M., Thompson, T.G., McPherson, J.D., Kote-Jarai, Z., Carpten, J.D., DiDonato, C.J., Ikeda, J-E., Surh, L., Wirth, B., Sargent, C.A., Ferguson-Smith, M.A., Fuerst, P., Moysis, R.K., Grady, D.L., Zerres, K., Korneluk, R., MacKenzie, A. and Wasmuth, J.J. (1994). A multicopy dinucleotide marker that maps close to the spinal muscular atrophy gene. *Genomics* 21: 394-402.
8. Brzustowicz, L.M., Lehner, T., Castilla, L.H., Penchaszadeh, G.K., Wilhelmsen, K.C., Daniels, R., Davies, K.E., Leppert, M., Ziter, F., Wood, D., Dubowitz, V., Zerres, K., Hausmanowa-Petrusewicz, I., Ott, J., Munsat, T.L. and Gilliam, T.C. (1990). Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. *Nature* 344: 540-541.
9. Church, D.M., Stotler, C.J., Rutter, J.L., Murrell, J.R., Trofatter, J.A., and Buckler, A.J. (1994). Isolation of genes from complex sources of mammalian genomic DNA using exon amplification. *Nature Genet.* 6, 98-105.

10. Clem, R.J., and Miller, L.K. (1994a). Induction and Inhibition of Apoptosis by Insect Viruses. *Apoptosis II: The Molecular Basis of Apoptosis in Disease*, Cold Spring Harbour Laboratory Press pp. 89-110.
11. Clermont, O., Burlet, P., Burglen, L., Lefebvre, S., Pascal, F., McPherson, J., Wasmuth, J., Cohen, D., Le Paslier, D., Weissenbach, J., Lathrop, M., Munnich, A., and Melki, J. (1994). Use of genetic and physical mapping to locate the spinal muscular atrophy locus between two new highly polymorphic DNA markers. *Am. J. Hum. Genet.* 54: 687-694.
12. Dana, S., and Wasmuth, J.J. (1982). Linkage of the leuS, emtB, and chr genes on chromosome 5 in humans and expression of human genes encoding protein synthesis components in human-Chinese hamster hybrids. *Somatic Cell Genet.* 8: 245.
13. Davis, L.G., Dibner, M.D. and Battey, J.F. (1986). *Basic Methods in Molecular Biology*. Elsevier, New York.
14. Dear, S., and Straden, R.A. (1991). A Sequence Assembly and Editing Program for Efficient Management of Large Projects. *Nucl. Acids. Res.* 19, 3907-3911.
15. DiDonato, C.J., Morgan, J., Carpten, J.D., Fuerst, P., Ingraham, S.E., Prescott, McPherson, J.D., Wirth, B., Zerres, K., Hurko, O., Wasmuth, i.i., Mendell, J.R., and Burghes, A.H.M. (1994). Association between Ag1-CA Alleles and Severity of Autosomal Recessive Proximal Spinal Muscular Atrophy. *Am. J. Hum. Genet.* (in press).
16. Dubowitz, V. (1978). *Muscle Disorders in Childhood*, W.B. Saunders Co. Ltd., East Sussex, pp. 146-190.
17. Dubowitz, V. (1991). Chaos in classification of the spinal muscular atrophies of childhood. *Neuromusc. Disord.* 1: 47-53.
18. Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem.* 132: 6-13.
19. Francis, M.J., Morrison, K.E., Campbell, L., Grewal, P.K., Christodoulo, Z., Daniels, R.J., Monaco, A.P., Frichauf, A.M., McPherson, J., Wasmuth, J. and Davies, K.E. (1993). A contig of non-chimeric YACs containing the spinal muscular atrophy gene in 5ql3. *Hum. Mol. Genet.* 2: 1161-1167.

20. Frisch, S.M., and Francis, H., (1994) Disruption of Epithelial Cell-Matrix Interactions Induces Apoptosis. *J. Cell Biol.*, 124, 619-626.
21. Garcia I. Martinou I Tsujimoto, Y. Martinou J.C. (1992) Prevention of Programmed Cell Death of Sympathetic Neuron by the bcl-2 proto-oncogene. *Science* 258:302-304.
22. Gilliam, T.C., Freimer, N.B., Kaufmann, C.A., Powhik, P.P., Bassett, A.S., Bengtsson, U., and Wasmuth, J.J. (1989). Deletion mapping of DNA markers to a region of chromosome 5 that cosegregates with schizophrenia. *Genomics* 5: 940-944.
23. Gilliam, T.C., Brzustowicz, L.M., Castillo, L.H., Lehner, T., Penchaszadeh, G.K., Daniels, R.J., Byth, B.C., Knowles, J., Hislop, J.E., Shapira, Y., Dubowitz, V., Munsat, T.L., Ott, J. and Davies, K.E. (1990). Genetic homogeneity between acute and chronic forms of spinal muscular atrophy. *Nature* 345: 823-825.
24. Gleeson, R., and Hillier, L. (1991). A Trace Display and Editing Program for Data from Fluorescence Based Sequencing Machines. *Nucl. Acids. Res.* 19, 6491-643, 47.
25. Hedrick, P.W. (1987). Gametic Disequilibrium Measures: Proceed with Caution. *Genetics* 117, 331-341.
26. Hockenberry, D., Nunez, G., Millman, C., Schreiber, R.D., and Korsmeyer, S.J. (1990) Bcl-2 is an Inner Mitochondrial Membrane That Blocks Programmed Cell Death. *Nature* 348, 334-336.
27. Hudson, T.J., Englestein, M., Lee, M.K., Ho, E.C., Rubenfield, M.J., Adams, C.P., Housman, D.E., and Dracopoli, N.C. (1992). Isolation and chromosomal assignment of 100 highly informative human simple sequence repeat polymorphisms. *Genomics* 13: 622-629.
28. Ioannou, P.A., Amemiya, C.T., Garnes, J., Droisel, P.M., Shizuya, H., Chen, C., Batzer, M.A., de Jong, P.J. (1994). A New Bacteriophage Pi-derived Vector for the Propagation of Large Human DNA Fragments. *Nature Genet.* 6, 84-89.
29. Jones, D.T., Taylor, W.R., and Thornton, J.M. (1994). A Model Recognition Approach to the Prediction of All-Helical Membrane Protein Structure and Topology. *Biochemistry* 33, 3038-3049.

30. Kley, P.W., Wang, C.H., Lien, L.L., Vitale, E., Pan, J., Ross, B.M., Grunn, A., Palmer, D.A., Warburton, D., Brzustowicz, L.M., Kunkel, L.M. and Gilliam, T.C. (1993). Construction of a yeast artificial chromosome contig spanning the spinal muscular atrophy disease gene region. *Proc. Natl Acad. Sci. USA* 90: 6801-6805.
31. Kouprina, N., Eldarov, M., Moyzis, R., Resnick, M. and Larionov, V. (1994). A model system to assess the integrity of mammalian YACs during transformation and propagation in yeast. *Genomics* 21: 7-17.
32. Larin, Z., Monaco, A.P. and Lehrach, H. (1991). Yeast artificial chromosome libraries containing large inserts from mouse and human DNA. *Proc. Natl. Acad Sci USA* 87: 4123-4127.
33. Lien, L.L., Boyce, F.M., Kley, P., Brzustowicz, L.M., Menninger, J., Ward, D.C., Gilliam, T. C., and Kunkel, L.M. (1991). Mapping of human microtubule associated protein 1B in proximity to the spinal muscular atrophy locus at 5q13.1 *Proc. Natl. Acad Sci.* 88: 7873-7876.
34. MacKenzie, A., Roy, N., Besner, A., Mettler, G., Jacob, P., Korneluk, R. and Surh, L. (1993). Genetic linkage analysis of Canadian spinal muscular atrophy kindreds using flanking microsatellite 5q13 polymorphisms. *Hum. Gen.* 90: 501-504.
35. Mankoo, B. S., Sherrington, R., De La Concha, A., Kalsi, G., Curtis, D., Melmer, G. and Gurling, H.M.D. (1991). Two microsatellite polymorphisms at the D5S39 locus. *Nucleic Acids Res.* 19: 1963.
36. McLean, M.D., Roy, N., MacKenzie, A.E., Salih, M., Burghes, A., Simard, L., Korneluk, R. G., Ikeda, J-E, and Surh, L. Two 5q13 simple tandem repeat loci are in linkage disequilibrium with type I spinal muscular atrophy. *Hum. Mol. Genet.*, In Press.
37. McLean M., Roy, N., Yalagi, G., Shutler, K., Tamai, Mahadevan S., Salih, M., Besner, A., Lefebvre, C., Kang, X., Aubry, H., Baird, S., Surh, L., Korneluk, R., MacKenzie, A. and Ikeda, J-E. Molecular genetics analysis of the spinal muscular atrophy region on 5Q13.1. Ikeda GenoSPERE Project. 31-35.
38. Melki, J., Abdelhak, S., Sheth, P., Bachelot, M.F., Burlét, P., Marcadet, A., Aicardi, J., Barois, A., Carriere, J.P., Fardeau, M., Fontan, D., Ponsot, G., Billsette, T., Angeline, C., Barbosa, C., Ferriere, G., Lanzi, G., Ottolini, A., Babron, M.C., Cohen, D., Hanauer, A., Colerget-Darpo, F., Lathrop, M., (1993) Refined Linkage Map of Chromosome 5 in the Region of the Spinal Muscular Atrophy Gene, *Genomics* 15:521-541.

39. Melki, J., Lefebvre, S., Burglen, L., Burlet, P., Clermont, O., Millasseau, P., Reboullet, S., Benichou, B., Zevianai, M., LePaslier, D., Cohen, D., Weissenbach J. and Munnich A., (1994). De novo and inherited deletions of the 5q13 region spinal muscular atrophies. *Science* 264, 1474-1477.
40. Meredith, J.E., Fazeli, B., and Schwartz, M.A. (1993). The Extracellular Matrix as a Cell Survival Factor. *Mol. Biol. Cell* 4, 953-961.
41. Munnich, A. and Frezal, J. (1990). Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. *Nature* 344: 767-768.
42. Melki, J., Lefebvre, S., Burglen, L., Burlet, P., Clermont, O., Millasseau, P., Reboullet, S., Benichou, B., Zevianai, M., LePaslier, D., Cohen, D., Weissenbach, J. and Munnich, A. (1994). De novo and inherited deletions of the 5q13 region in spinal muscular atrophies. *Science* 264: 1474-1477.
43. Neil, D.L., Villasante, A., Fisher, R.B., Vetrie, D., Cox, B. and Tyler-Smith, C. (1990). Structural instability of human tandemly repeated DNA sequences cloned in yeast artificial chromosome vectors. *Nucleic Acid Res.* 18: 1421-1428.
44. Nelson, D.L., Brownstein, B.H. (eds) (1993). YAC libraries: A users guide. W.H. Freeman and Company, New York pp. 86-89.
45. Meakin, .S.O., and Shooter, E.M. (1992). The Nerve Growth Factor Family of Receptors. *TINS* 9, 323-331.
46. Oppenheim, R.W. (1991). Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14, 5453-501.
47. Oshima, A., Kyle, J.W., Miller, R.D., Hoffmann, Powell, P.P., Grubb, J.H., Sly, W.S., Tropak, M., Guise, K.S., and Gravel, R.A. (1987). Cloning, sequencing and expression of cDNA for human beta-glucuronidase. *Proc. Natl. Acad. Sci. USA.* 84: 685-689.
48. Podolsky, L., Tsilfidis, C., Baird, S., Korneluk, R.G., and MacKenzie, A.E. (1994). An Empiric Comparison of Linkage Disequilibrium Parameters in Disease Gene Localization: The Myotonic Dystrophy Experience. *Am. J. Hum. Genet.* 55, A932.
49. Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., and Jacobson, M.D. (1993). Programmed Cell Death and the Control of Cell Survival: Lessons from the Nervous System. *Science* 262:695-698.

50. Roy, N., McLean, M., Johnston, A., Lefebvre, C., Salih M., Yaraghi, Z., Ikeda, J.E., Korneluk, R.G., MacKenzie, A.E. (1994) Refined physical map of the spinal muscular atrophy gene region at 5q13 based on YAC and cosmid contiguous arrays, *Genomics*, (submitted).
51. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
52. Sarnat, H.B. (1992) Cerebral dysgenesis: Embryology and clinical expression, Oxford University Press, p. 107.
53. Scherer, S. and Tsui, L.-C. (1991). Adolph, K.W., ed Cloning and analysis of large DNA molecules. Advanced Techniques in Chromosome Research. Dekker, New York. pp.33-72.
54. Soares, V.M., L.M., Kleyn, P.W., Knowles, J.A., Palmer, D.A., Asokan, S., Penchaszadeh, G.K., Munsat, T.L. and Gilliam, T.C. (1993). Refinement of the spinal muscular atrophy locus to the interval between D5S435 and MAPIB. *Genomics* 15: 365-371.
55. Sherrington, R., Melmer, G., Dixon, M., Curtis, D., Mankoo, B., Kalsi, G. and Gurling, H. (1991). Linkage disequilibrium between two highly polymorphic microsatellites. *Am. Hum. Genet.* 49: 966-971.
56. Shutler, G., Korneluk, R.G., Tsilfidis, C., Mahadaven, M., Bailly, J., Smeets, H., Jansen, G., Wieringa, B., Lohman, F., Asanidis, C., and de Jong, P.J. (1992). Physical mapping and cloning of the proximal segment of the Myotonic Dystrophy Gene Region. *Genomics* 13: 513-525.
57. Stahl, N., Boulton, T.G., Farruggella, T., Ip. N.Y., Davis, Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J.N., and Yancopoulos, G.D. (1994). Association and Activation of Jak-Tyk Kinases by CNTF-LIF-OSM-IL-6 S Receptor Components. *Science* 263, 92-95.
58. Stallings, R.L., Doggett, N.A., Okumura, K. and Ward, D.C. (1992). Chromosome 16-specific repetitive DNA sequences that map to chromosomal regions known to undergo breakage/rearrangement in leukemia cells. *Genomics* 13: 332-338.
59. Tagle, D.A., Collins, F. S. (1992). An optimized Alu-PCR primer pair for human-specific amplification of YACs and somatic cell hybrids. *Hum.Molec. Genet.* 1: 121-122.

60. The European Polycystic Kidney Disease Consortium (1994). The polycystic kidney disease gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. *Cell* 77: 881-894.
61. Thompson, T.G., Morrison, K.E., Kleyn, P., Bengtsson, U., Gilliam, T.C., Davies, K.E., Wasmuth, J.J. and McPherson, J.D. (1993). High resolution physical map of the region surrounding the spinal muscular atrophy gene. *Hum. Mol. Genet.* 2: 1169-1176.
62. van der Steege, G., Cobben, J-M., Osinga, J., Schaffer, H., van Ommen, G-J.B., and Buys, C.H.C.M. A sublocus of the multicopy microsatellite marker CMS1 maps proximal to SMA as shown by recombinant analysis. *Genomics*, submitted.
63. Wirth, B., Voosen, B., Röhrig, D., Knapp, M., Piechaczek, B., Rudnik-Schöneborn. and Zerres, K. (1993). Fine mapping and narrowing of the genetic interval of the spinal muscular atrophy region by linkage studies. *Genomics* 15: 113-118.
64. Wirth, B., Pick, E., Leuter, A., Dadze, A., Voosen, B., Knapp, M., Piechaczak-Wappenschmidt, B., Rudnik-Schöneborn, S., Schonling, J., Cox, S., Spurr, N.K. and Zerres, K. (1994). Large linkage analysis in 100 families with autosomal recessive spinal muscular atrophy (SMA) and 11 CEPH families using 13 polymorphic loci in the region 5q11.2-q13.3. *Genomics* 20: 84-93.
65. Warrington, J.A., Bailey, S.K., Armstrong, E., Aprelikova, O., Alitolo, K., Dolganov, G.M., Wilcox, A.S., Sikela, J.M., Wolfe, S.F., Lovett, M., and Wasmuth, J.J. (1992). A radiation hybrid map of 18 growth factor, growth factor receptor, hormone receptor, neurotransmitter receptor genes on the distal region of the long arm of chromosome 5. *Genomics*. 13: 803-808.
66. Yaraghi, Z., Kang, X, Ikeda, J-E. and MacKenzie, A. *Hum. Mol. Genet.*, In Press.
67. Yaraghi, Z., McLean, M., Roy, N., Surh, L., Ikeda, J-E., and MacKenzie, A.E. A recombination event occurring within the two complex 5q13.1 simple tandem repeat polymorphisms suggests a telomeric mapping of spinal muscular atrophy. *Genomics*. Submitted.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A human gene which maps to the SMA containing region
5 of chromosome 5q13, said gene comprising exons 1 through 17 of approximately 5.5 kb and having a restriction map for exons 2 through 11 as shown in Figure 8.
2. A human gene which maps to the SMA containing region
10 of chromosome 5q13, said gene comprising exons 1 through 17 of approximately 5.5 kb and having a restriction map for exons 2 through 16 as shown in Figure 9D.
3. A human gene of claim 1 or 2 wherein exons 5 through
15 16 code for the NAIP protein having an amino acid sequence biologically functionally equivalent to the amino acid sequence of Sequence ID No. 2.
4. A human gene of claim 1 or 2 wherein exons 5 through
20 16 have a cDNA sequence biologically functionally equivalent to the cDNA sequence of Sequence ID No. 1.
5. A purified nucleotide sequence comprising genomic
DNA, cDNA, mRNA, anti-sense DNA or homologous DNA
25 corresponding to the cDNA sequence of Sequence ID No. 1.
6. A DNA molecule comprising a DNA sequence of Sequence ID No. 1.
- 30 7. A DNA molecule comprising a DNA sequence coding for the NAIP protein having Sequence ID No. 2.
8. A purified DNA sequence consisting essentially of DNA Sequence ID No. 1.
- 35 9. A purified DNA sequence consisting essentially of a DNA sequence coding for amino acid Sequence ID No. 2.

10. A purified DNA sequence comprising at least 18 sequential bases of Sequence ID No. 1.
11. A DNA probe comprising a DNA sequence of claim 10.
- 5 12. A PCR primer comprising a DNA sequence of claim 10.
13. A DNA hybridization molecule comprising a DNA sequence of claim 10.
- 10 14. A purified DNA sequence of claims 10, 11, 12 or 13 wherein said DNA sequence is selected from exons 1 through 4 and 17 of Table 4.
- 15 15. A purified DNA sequence of claims 10, 11, 12 or 13 wherein said DNA sequence is selected from exons 5 through 16 of Table 4.
- 20 16. A purified DNA sequence of claim 10, 11, 12 or 13 selected from the group of DNA sequences consisting of exon sequences 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17 of Table 4.
- 25 17. A purified DNA sequence of claim 16 wherein the DNA sequence is selected from exons 4, 5, 6 and 7.
18. A purified DNA sequence of claim 16 wherein the DNA sequence is selected from exons 5 and 6.
- 30 19. Use of DNA sequences of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 in the construction of a cloning vector or an expression vector.
20. NAIP protein encoded by a DNA sequence of claims 1, 35 2, 3, 4, 5, 6, 7, 8, or 9.

21. A 15 amino acid fragment of NAIP protein encoded by 45 sequential bases of the DNA sequence of claim 10.
22. NAIP protein comprising an amino acid sequence 5 biologically equivalent to the amino acid sequence of Sequence ID No. 2.
23. NAIP protein consisting essentially of the amino acid sequence of Sequence ID No. 2.
- 10 24. NAIP protein fragment comprising at least 15 sequential amino acids of Sequence ID No. 2.
25. NAIP protein fragment comprising an amino acid 15 sequence selected from the group of polypeptides encoded by one of exons 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16.
26. NAIP protein fragment of claim 25 wherein selected 20 polypeptides are those encoded by exons 5, 6, 7, 8, 9, 10, 11 or 12.
27. NAIP protein fragment of claim 25 wherein selected polypeptides are those encoded by exons 5 and 6.
- 25 28. Use of amino acid sequences of claims 20, 21, 22, 23, 24, 25 or 26 in production of hybridomas.
29. A method for analyzing a biological sample to 30 diagnose presence or absence of a gene encoding NAIP protein, said method comprising:
- i) providing a biological sample derived from the SMA containing region q13 of human chromosome 5;
 - ii) conducting a biological assay to determine 35 presence or absence in said biological sample of at least a member selected from the group consisting of:

- a) NAIP DNA Sequence ID No. 1, and
- b) NAIP protein Sequence ID No. 2.

30. A method of claim 29 for diagnosing a human's risk
5 of developing SMA wherein step ii) mutations in said
sequence of group a) or b) are assayed.

31. A method of claim 30 wherein presence or absence of
exons 5 and 6 of group a) are assayed.

10

32. A method of claim 31 further comprising determining
intact gene copy number in chromosome 5 of a gene
encoding said NAIP protein.

15 33. A method of claim 29, 30, 31 or 32 wherein said
biological assay comprises an assay selected from the
group consisting of DNA hybridization, restriction enzyme
analysis, PCR amplification, mRNA detection and DNA
sequencing.

20

34. A method of claim 29, 30, 31 or 32 wherein said
biological assay comprises PCR amplification of exon
regions 5 and 6 by use of PCR primers selected from the
5' region of exon 5 and 3' region of exon 6.

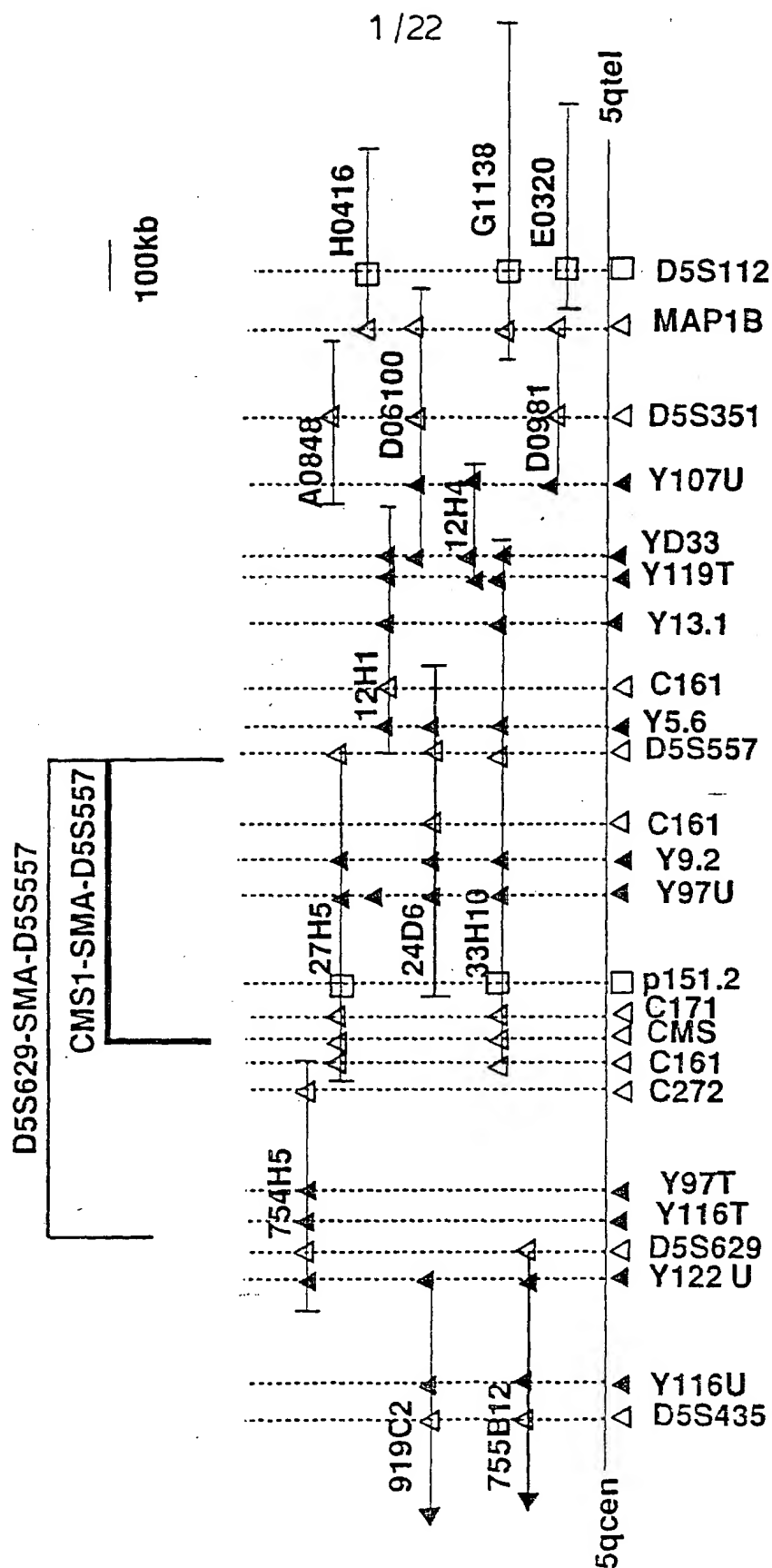
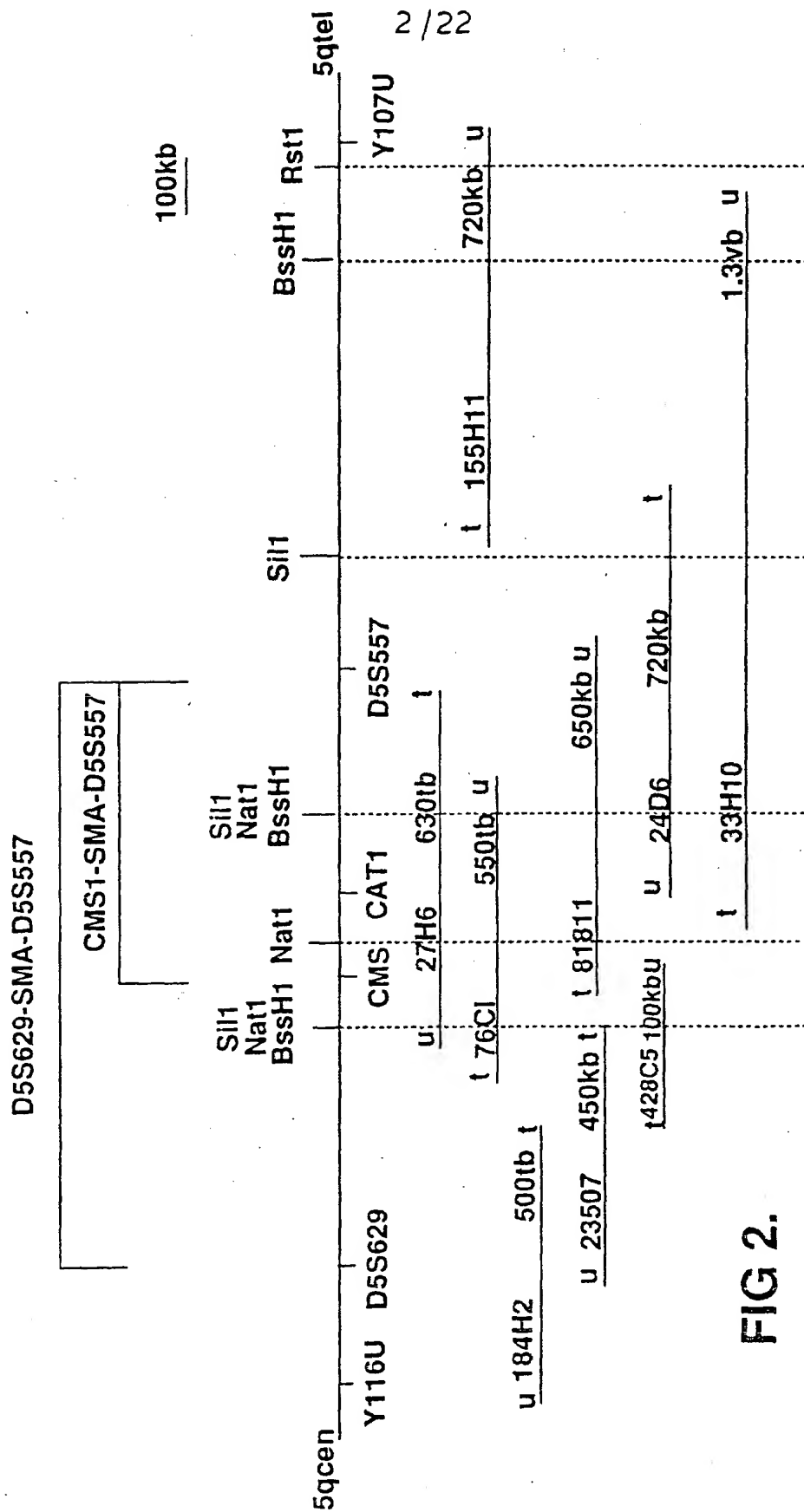


FIG 1.



3/22

108F4
126H11
123F6
40G1
58G12
192F7
250B6

10 12 12 15 12 10 7

FIG.3B.

G
G
D06100
12H1
12H4
24D6
27H5
33H10
76C1
155H11
184H2
235B7
81B11

15

12 7 15 15

FIG.3A.

6 6
7 7
9 6
11 11
12 12
15 15

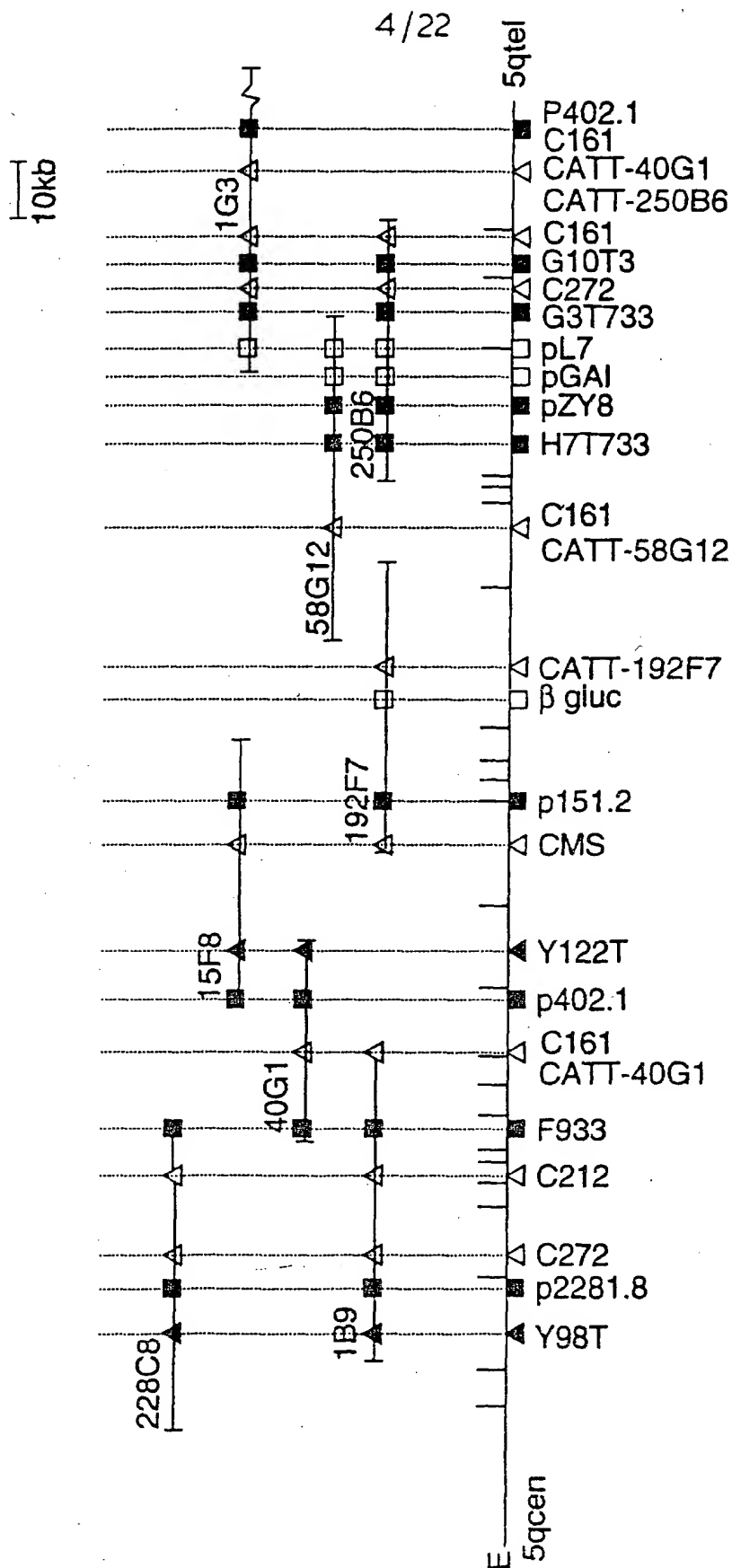


FIG.4

5/22

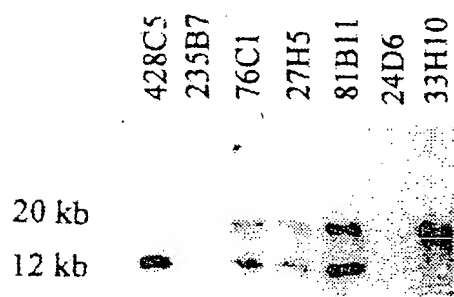


FIG.5A.

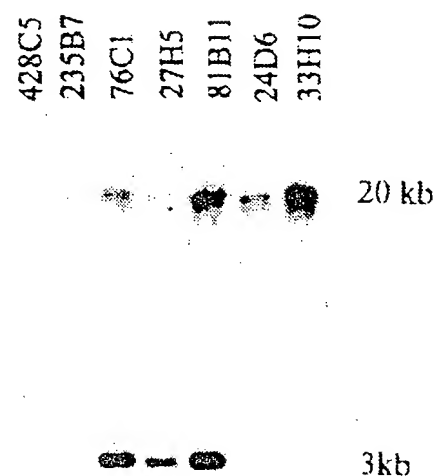


FIG.5C.

6/22

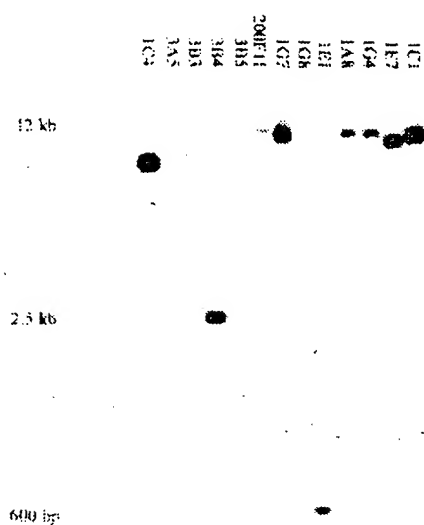


FIG. 5B.

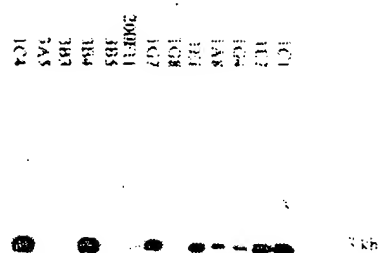


FIG. 5D.

7 / 22

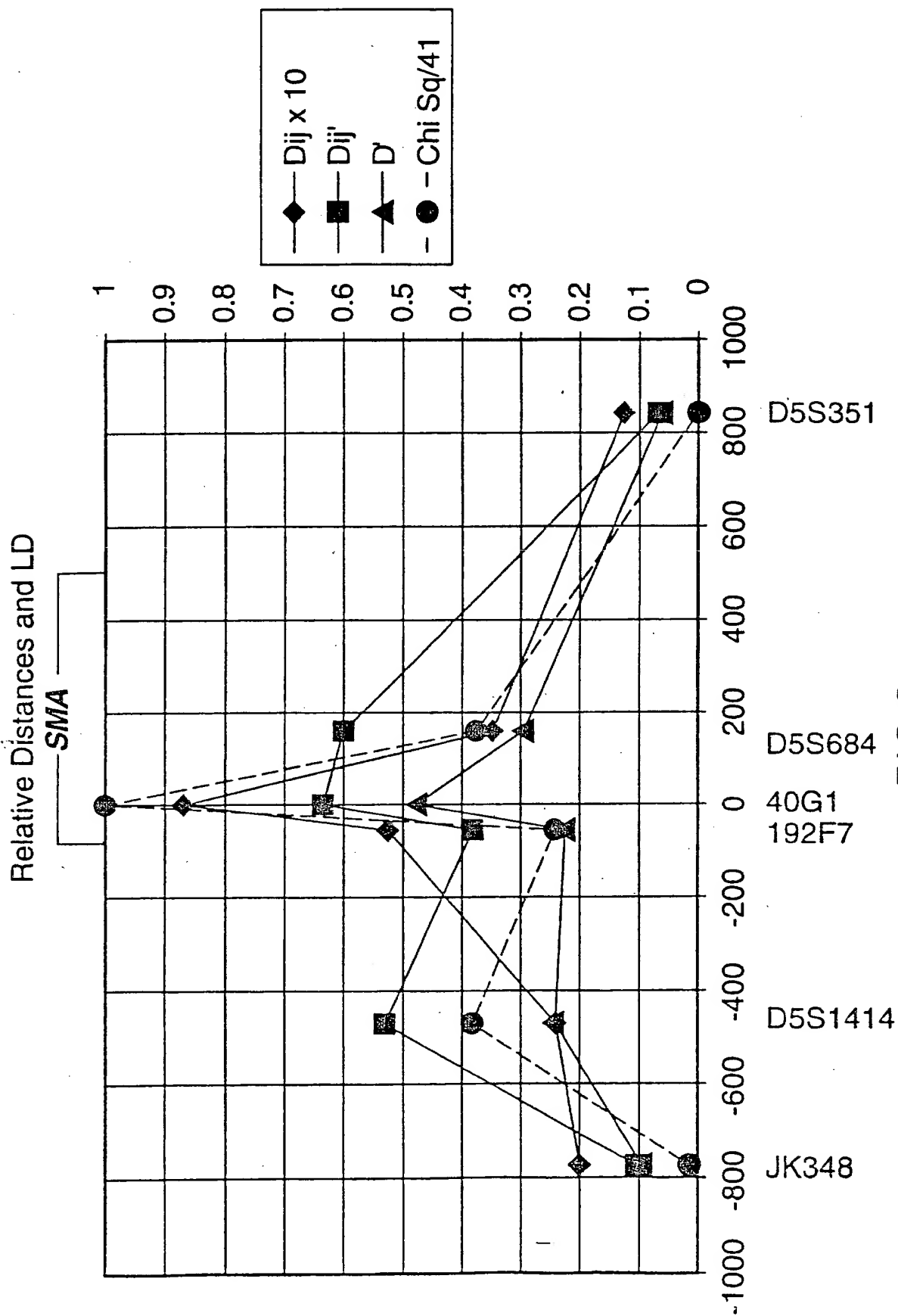


FIG. 6

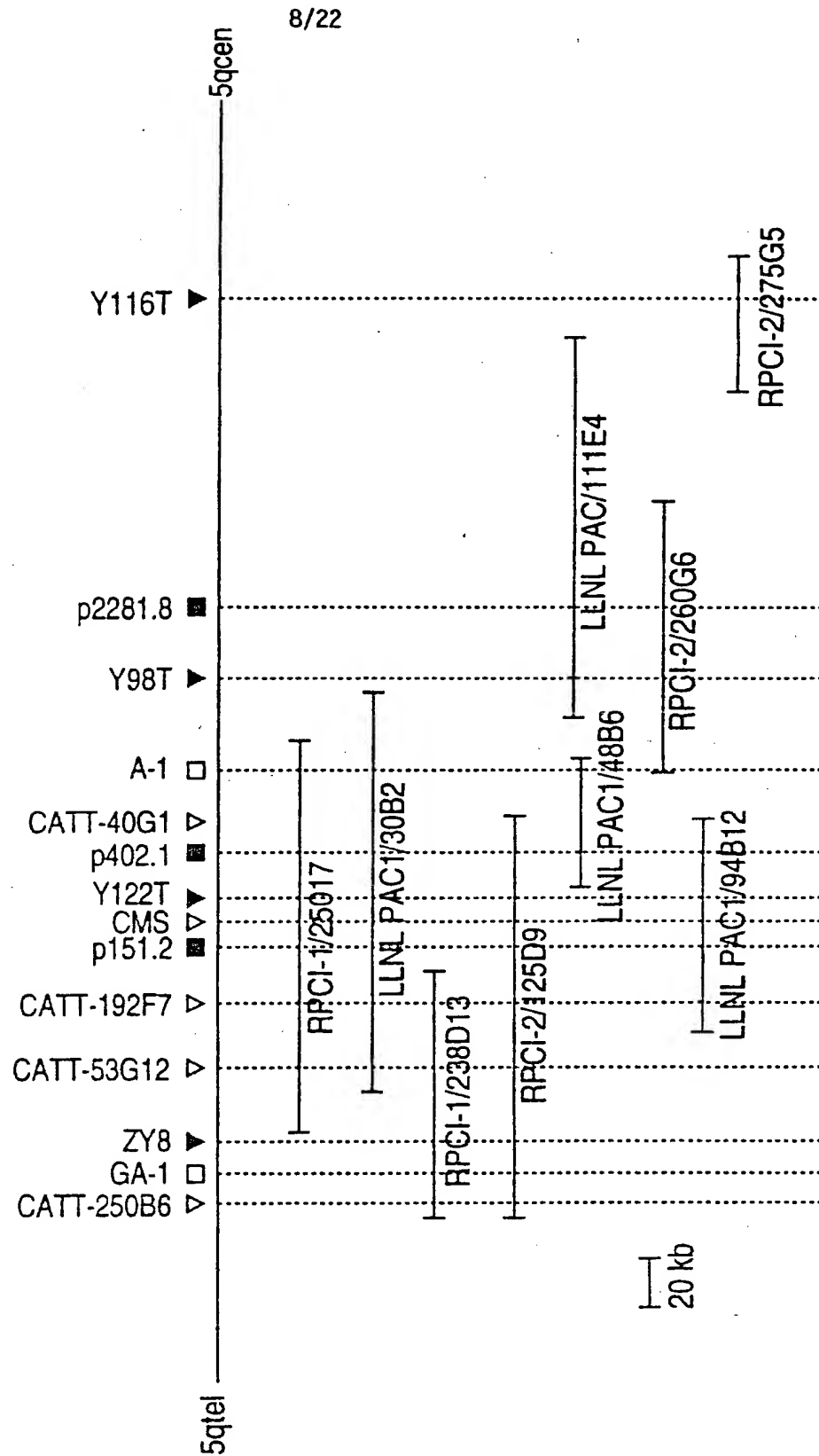


FIG.7

SUBSTITUTE SHEET (RULE 26)

2kb

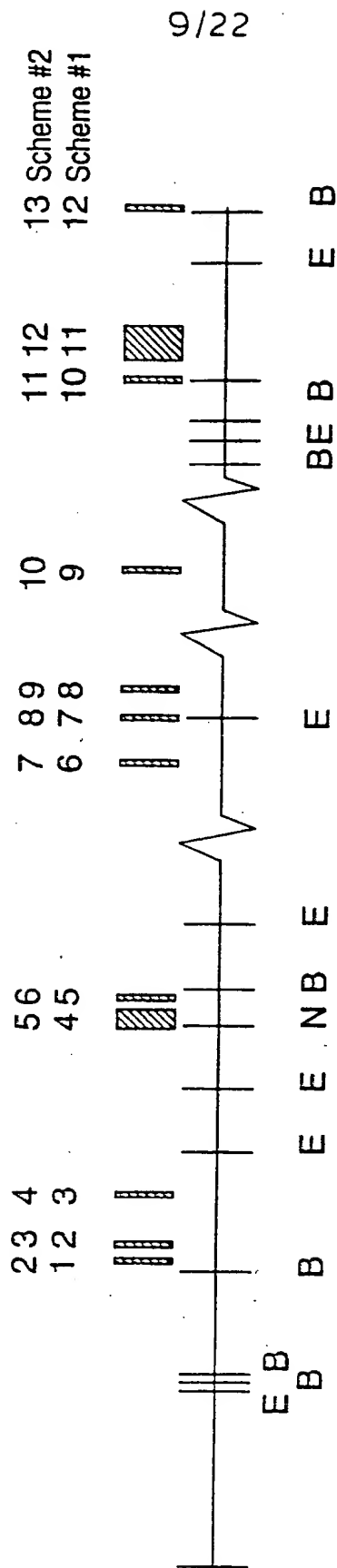


FIG.8

10/22

Correlation of Type I SMA 5q13.1
marker linkage disequilibrium with
5q13.1 physical map

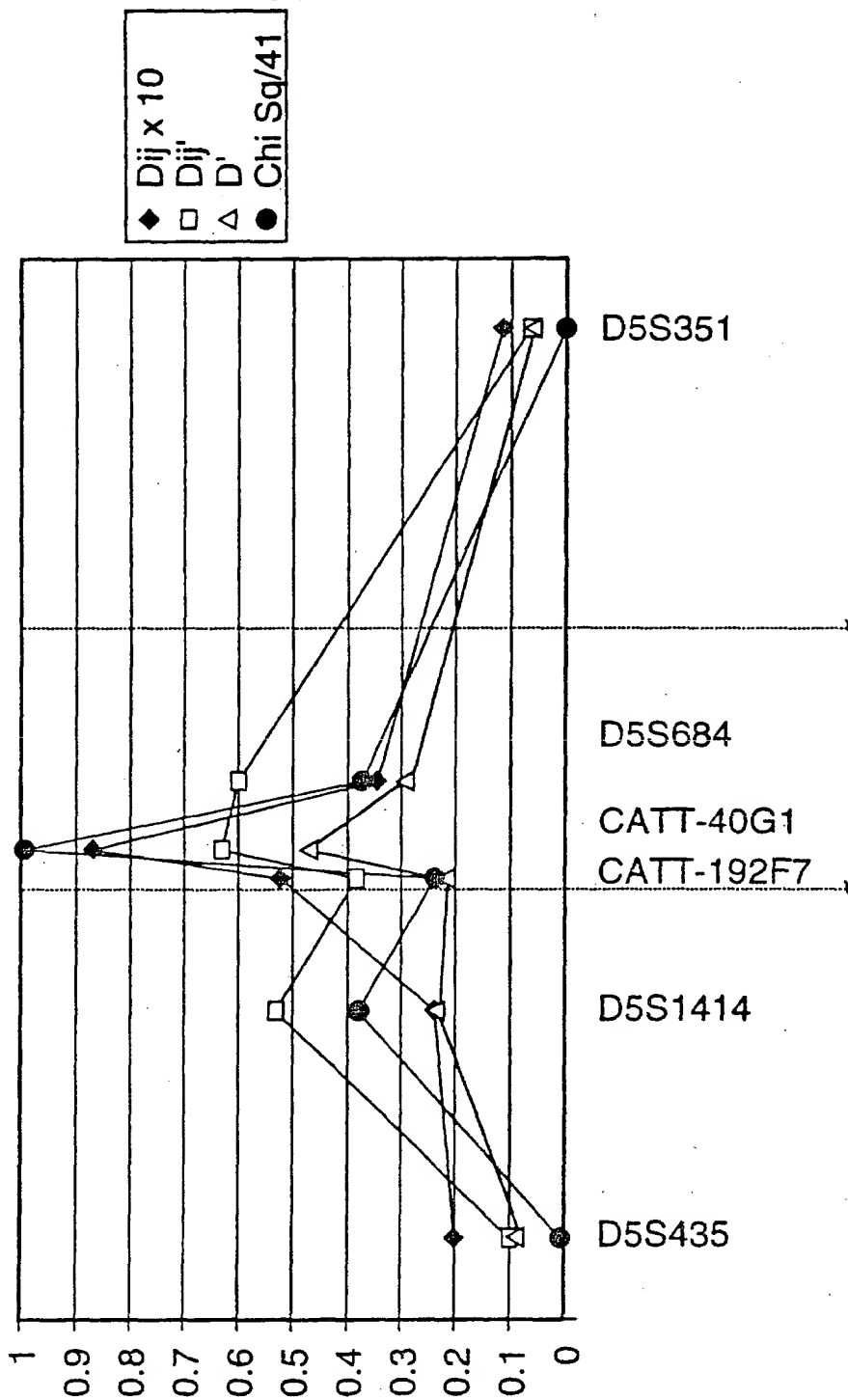


FIG. 9A

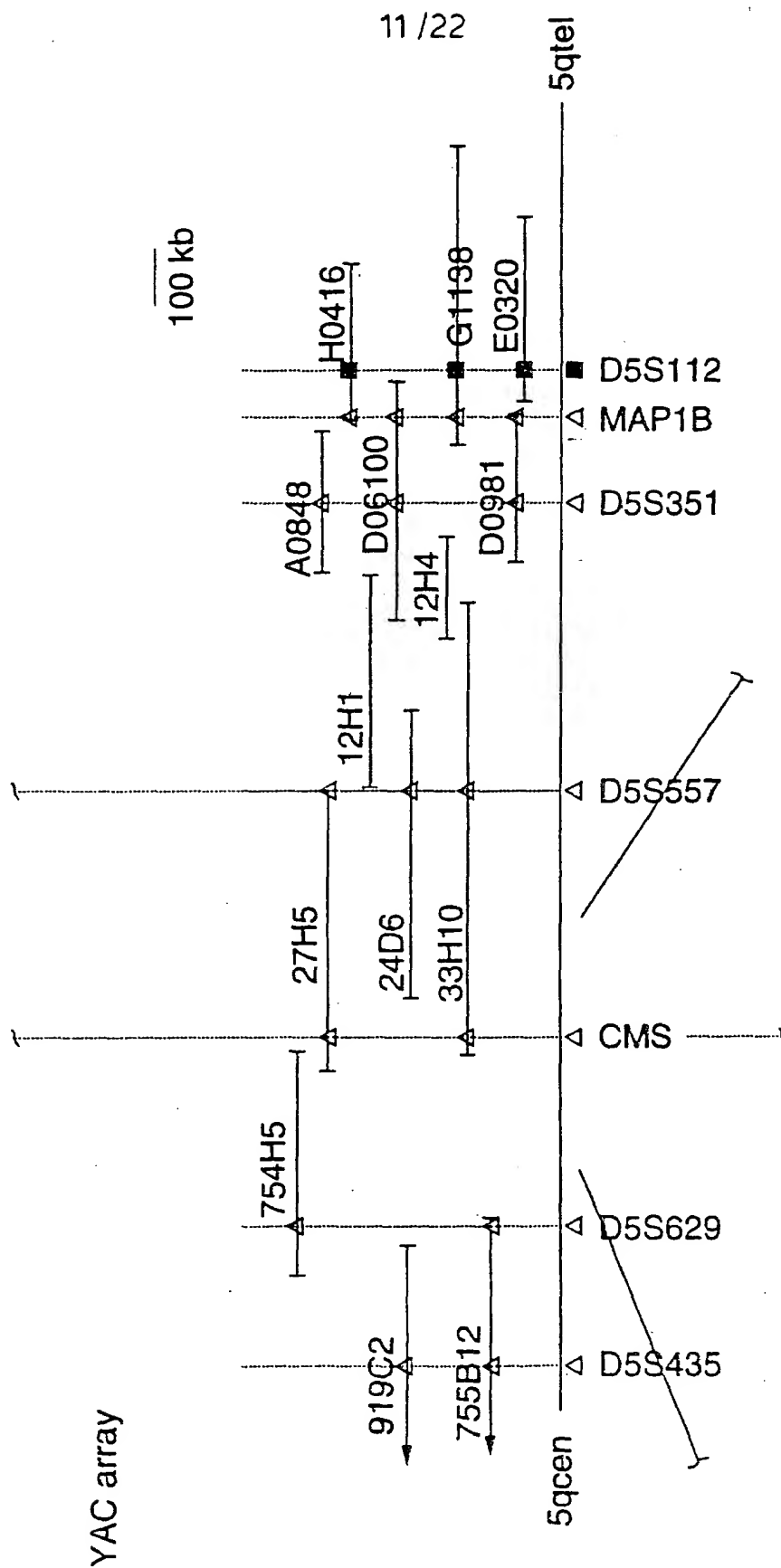


FIG.9B

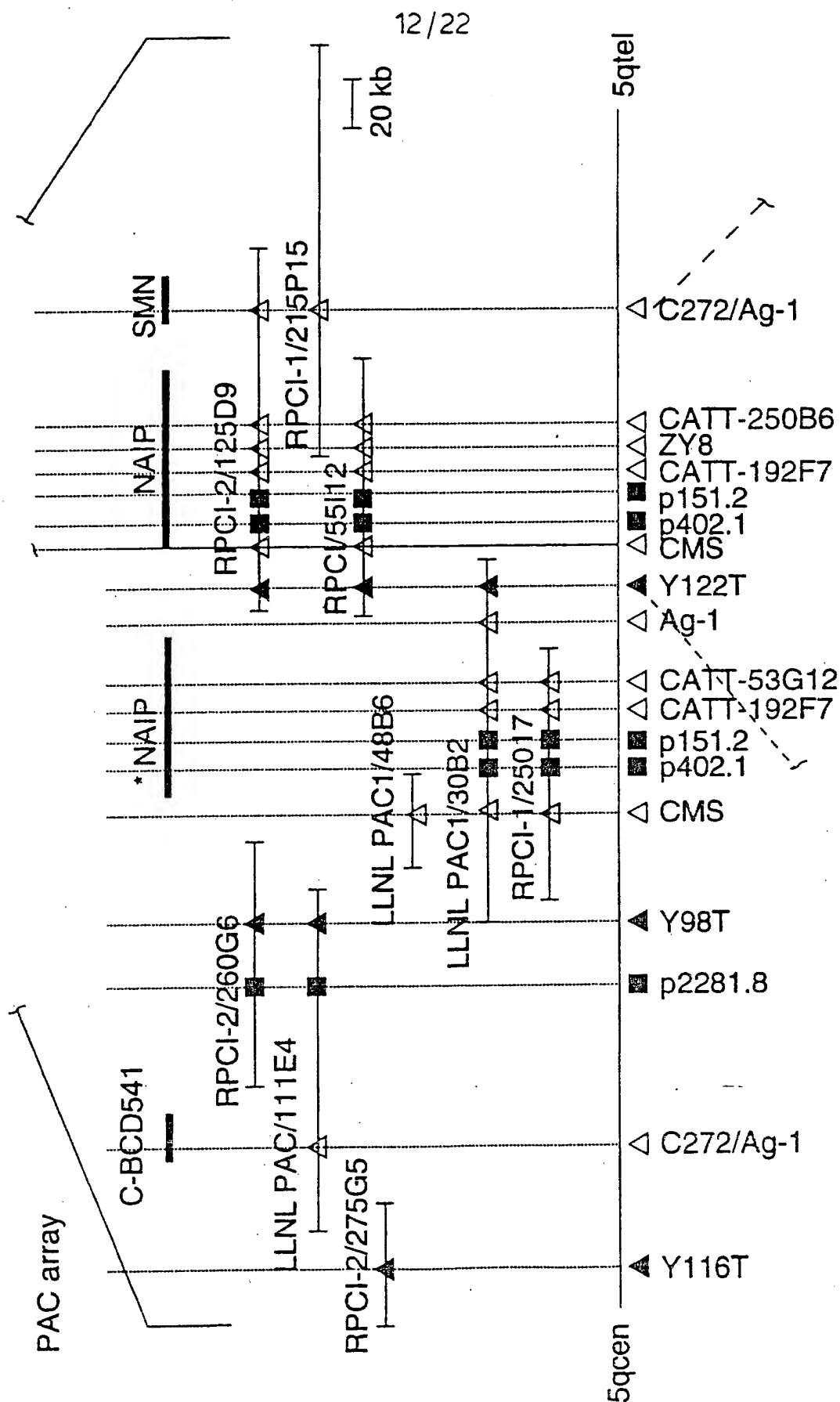


FIG.9C

13/22

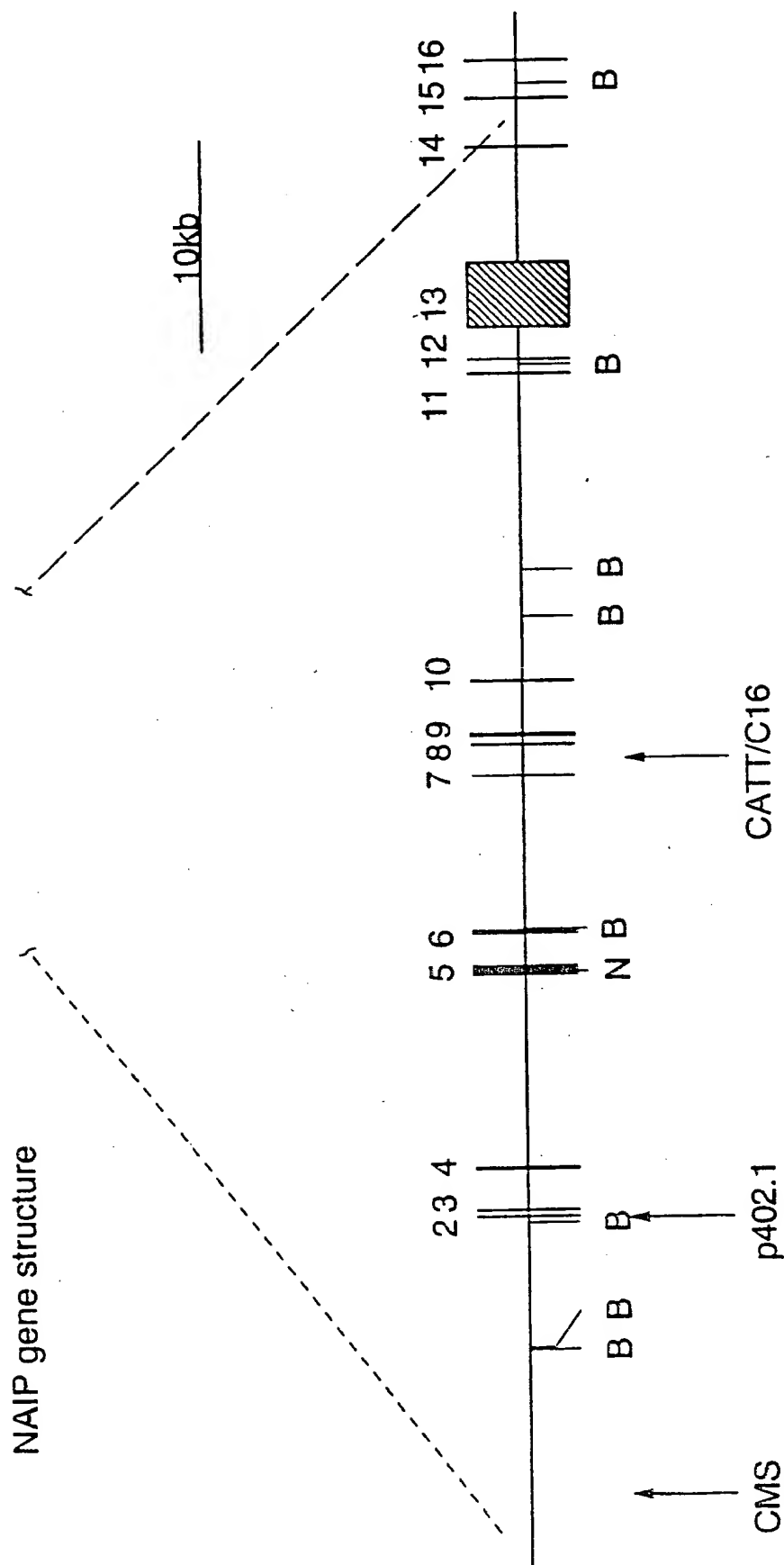


FIG.9D

14/22

Exon content of PAC, cDNA and RT-PCR clones

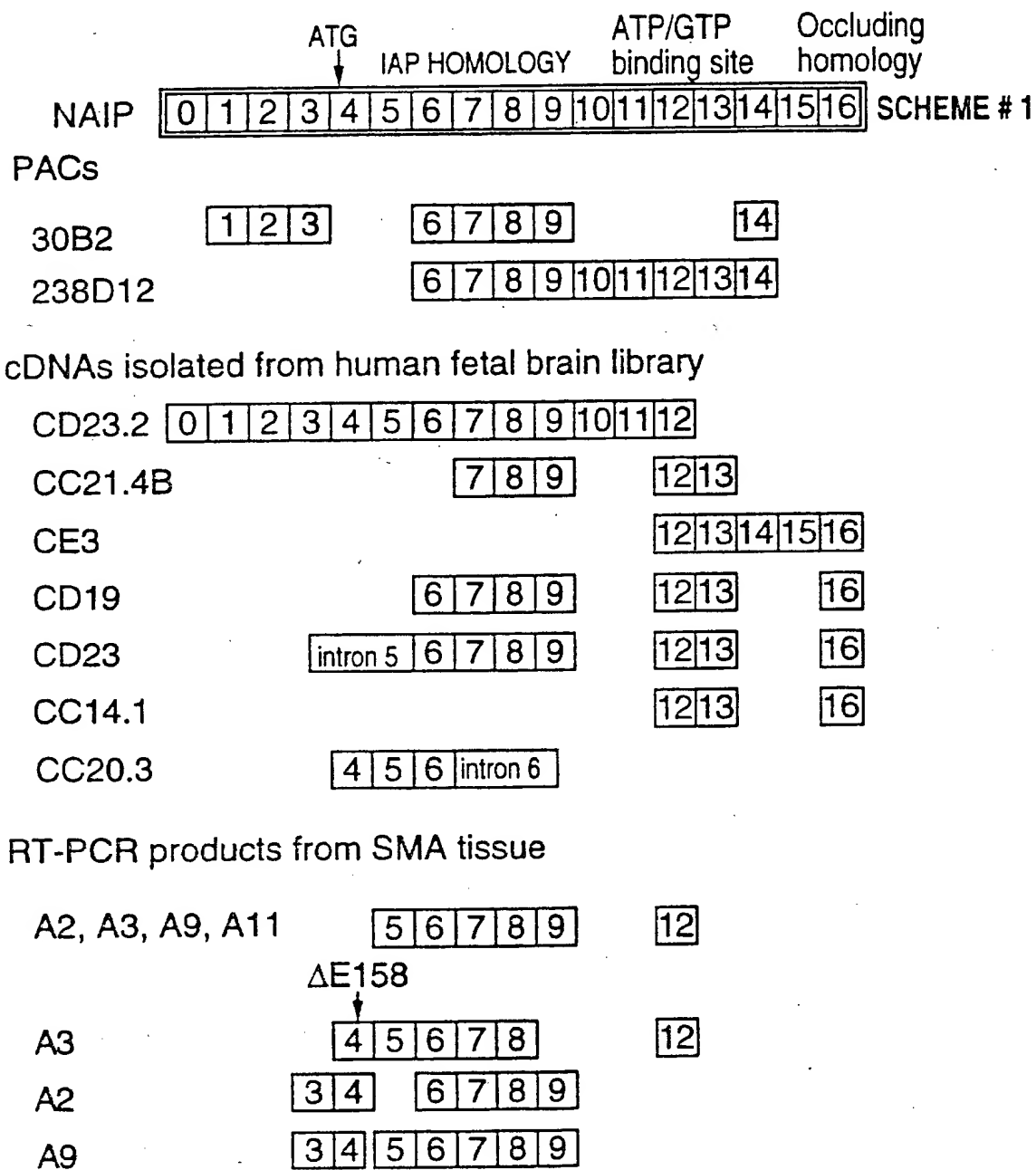


FIG.10

NAIP gene structure

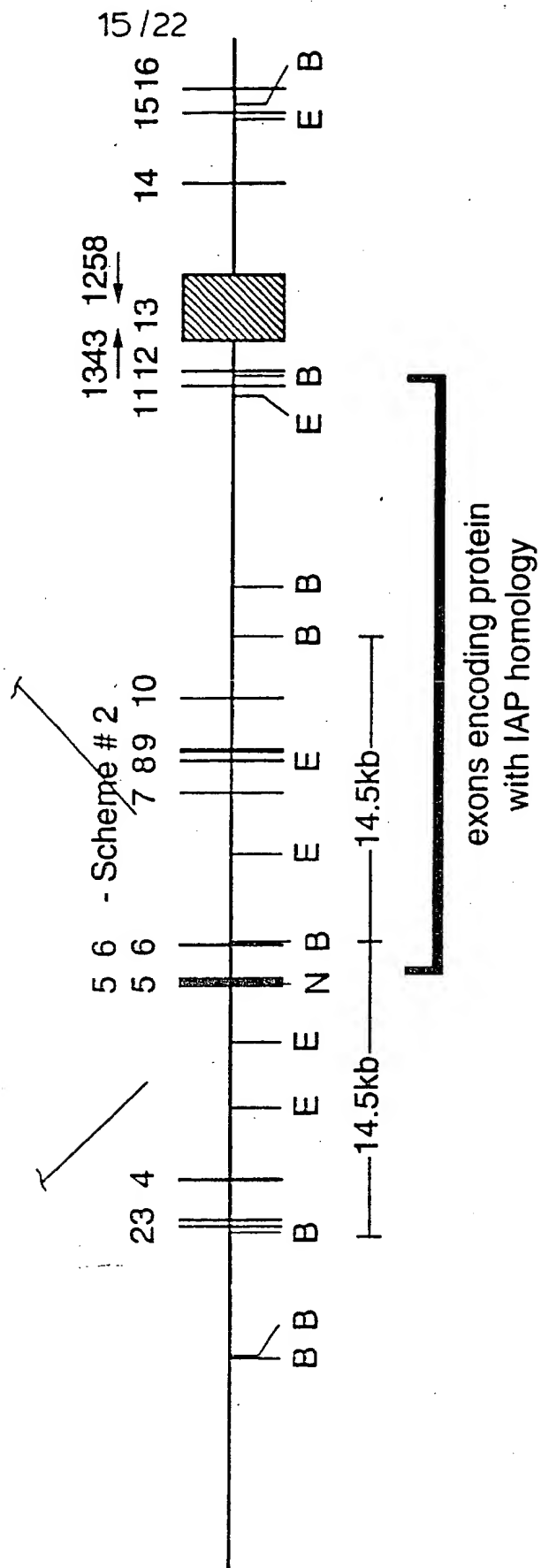


FIG.11A

16/22

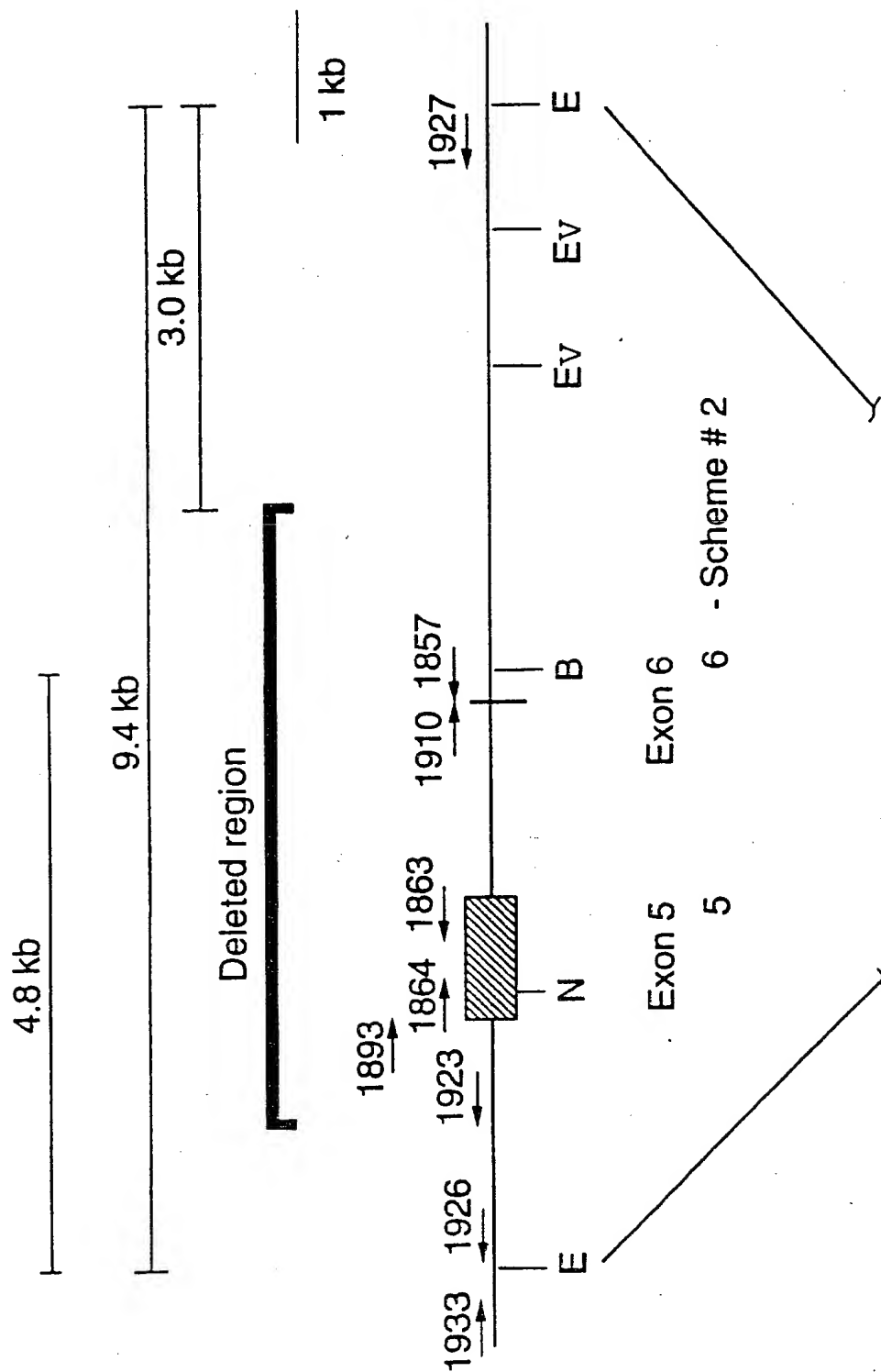


FIG.11B

17/22

10 kb

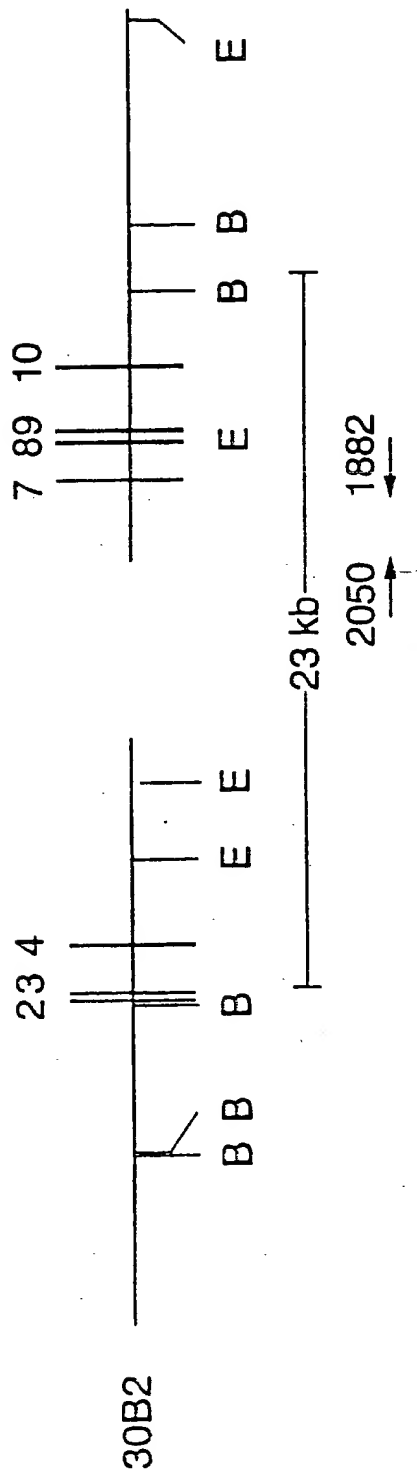


FIG. 11C

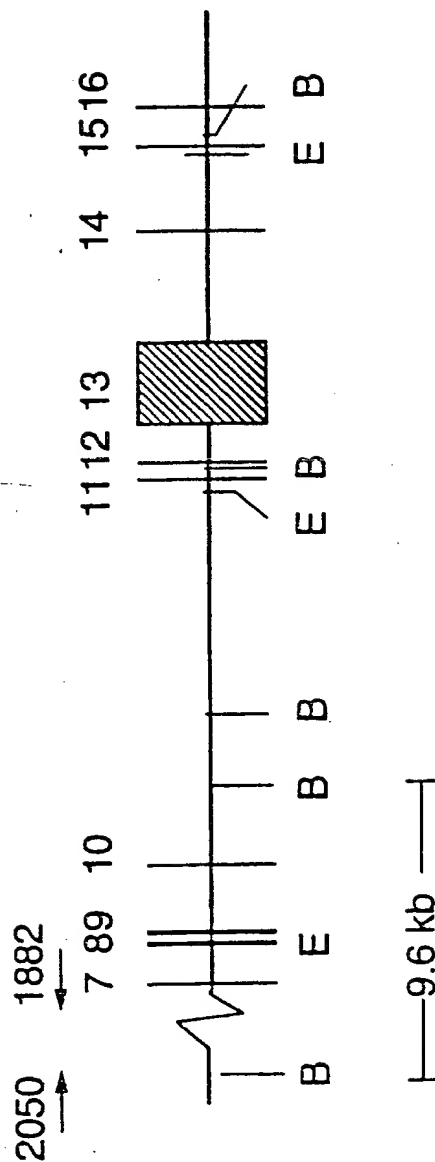


FIG. 11D

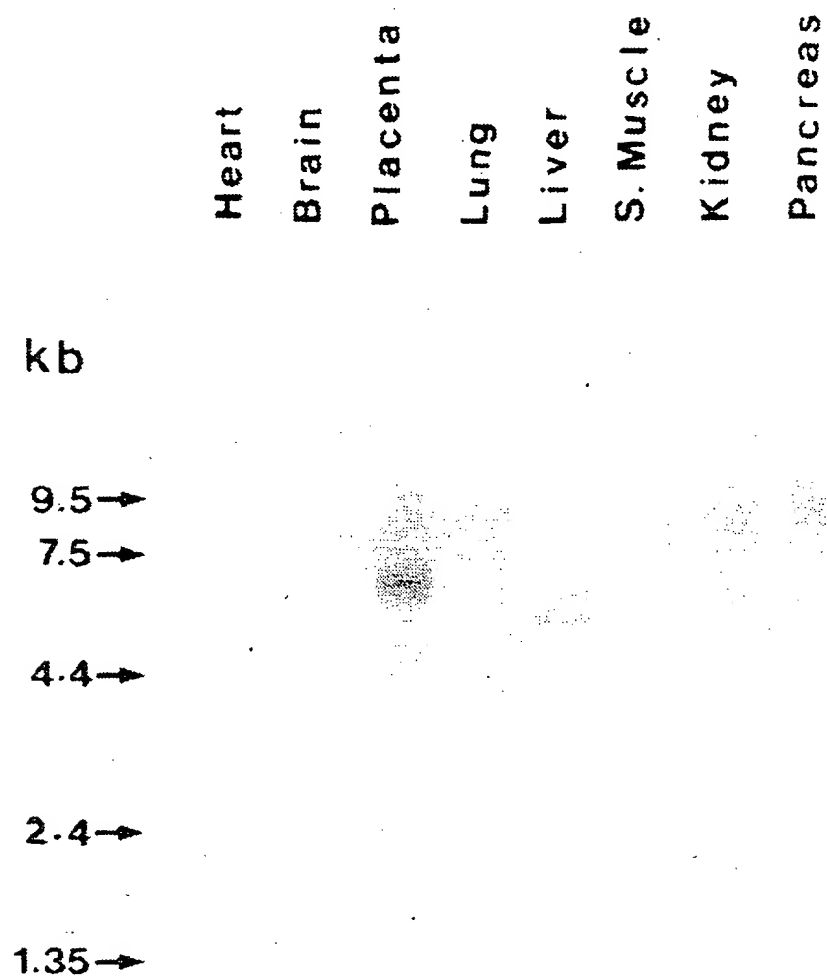
18/22

Splice Junctions of Exons of NAIP Mapping to PAC125D9

Exon #	3' Splice Junction	5' Splice Junction
0		
1	atgaag/ACAAAA	TCATCT/gtatcc
2	tgcgag/ACGACG	GCAAAG/gtgagt
3	ttttag/TCCTT	CCTCAG/gtaagt
4	tttcag/AAAATG	TTACAG/gtacct
5	atatag/GTAAAC	
6	ttacag/ATGTGA	ATAACG/gtaatg
7	cctcag/GGAGAA	CATCAG/gtaaaa
8	ttccag/CTTATT	ACACAG/gtgagt
9	ttttag/GTATAA	TCCCAA/gtgagt
10	acttag/TTGTCC	TTACTG/gtaatg
11	tcaaag/GAAACC	TGCCAG/gtaaga
12	ccacag/AAATGG	CACAAG/gtatac
13	ttccag/ACCAAA	AACTAG/gtaagg
14	tcatag/TTGCCA	AATTTG/gtatgt
15	ctgcag/CCTACA	AAATTG/gtgagc

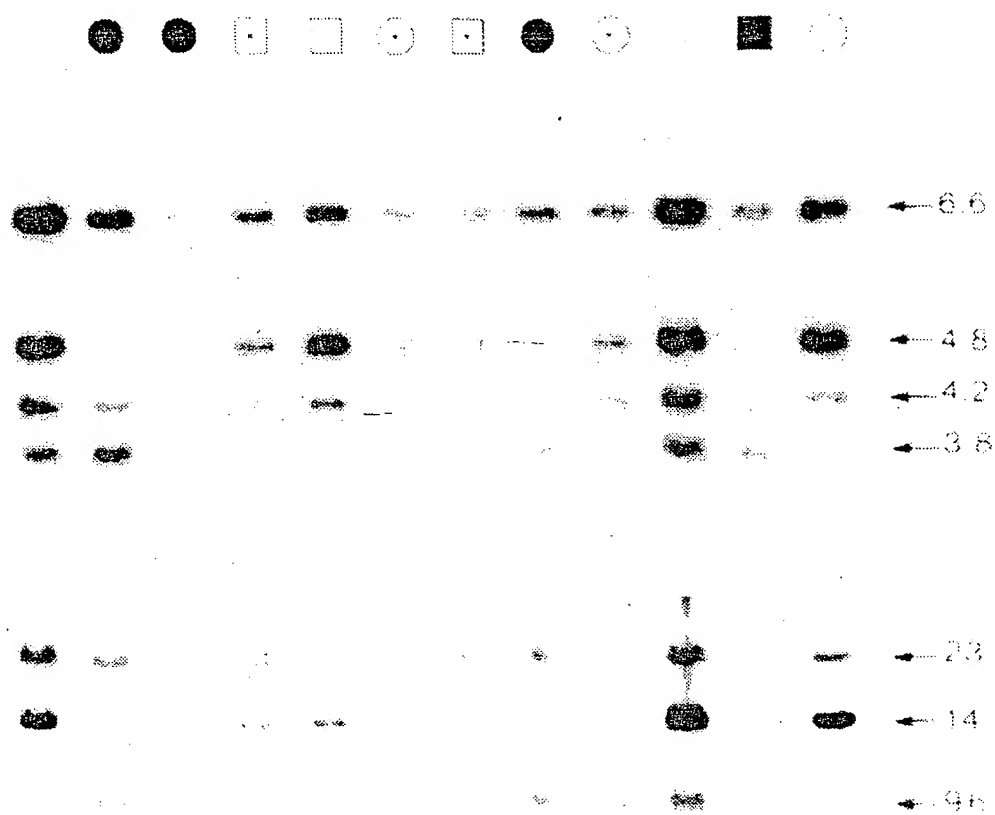
FIG.12

19/22
FIG.13.



20 / 22

FIG.14.

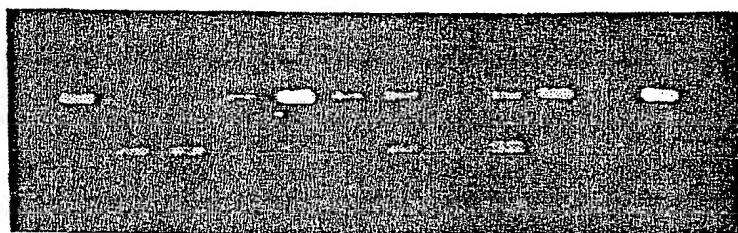
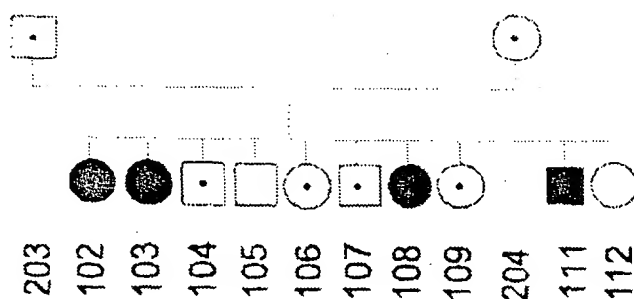


21 / 22

FIG.15A.

Family 24561

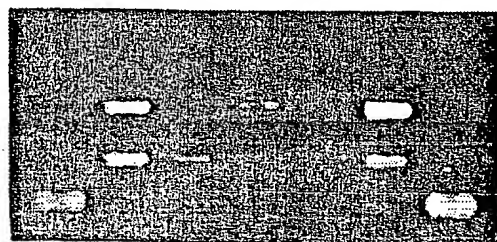
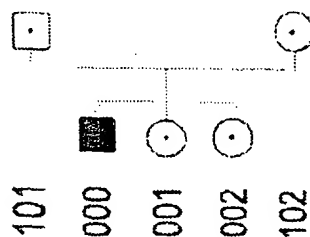
SCHEME 2.



← 435 bp
← 241 bp

FIG.15B.

Family 21470



← 435 bp
← 241 bp

22 / 22

FIG.16A.

spinal cord lymphoblast

n n a1 a2 a3 a4 n a5 a6 a7

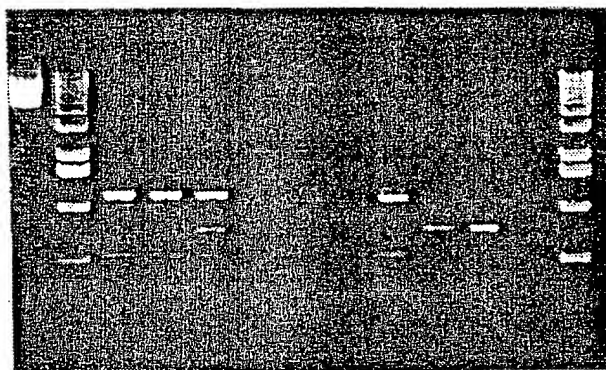


FIG.16B.

spinal cord lymph.

n n n a7 a1 a2 a3 n a7

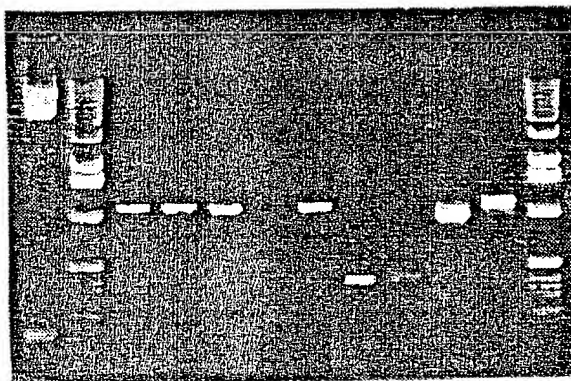
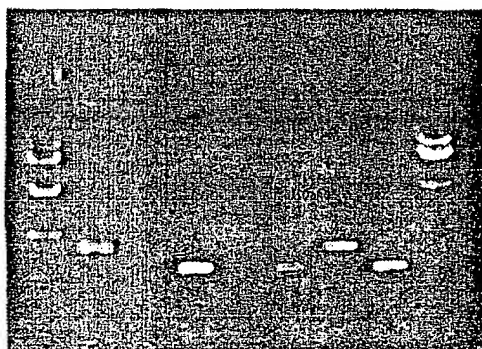


FIG.16C.

sp. cord lym.fib.

n n a2 a3 a9 a10 a11



INTERNATIONAL SEARCH REPORT

International Application No

PC/CA 95/00581

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/63 C12N15/81 C12Q1/68 C07K14/47
G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GENOMICS (1993), 15(2), 365-71 CODEN: GNMCEP; ISSN: 0888-7543, 1993</p> <p>SOARES, VERA M. ET AL 'Refinement of the spinal muscular atrophy locus to the interval between D5S435 and MAP1B' cited in the application</p> <p>see abstract</p> <p>see page 365, right column, paragraph 4</p> <p>see page 370, left column, last paragraph</p> <p>- right column, paragraph 2</p> <p style="text-align: center;">--- -/--</p>	1-34

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

31 January 1996

Date of mailing of the international search report

29.02.1996

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 95/00581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HUM. MOL. GENET. (1993), 2(8), 1161-7 CODEN: HMGEES; ISSN: 0964-6906, 1993 FRANCIS, MICHAEL J. ET AL 'A contig of non-chimeric YACs containing the spinal muscular atrophy gene in 5q13' cited in the application see abstract see page 1161, left column, paragraph 3 - right column, paragraph 1 see page 1164, right column, paragraph 2 - page 1166, left column, paragraph 1 ---</p>	1-19, 29-34
A	<p>44TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HUMAN GENETICS, MONTREAL, QUEBEC, CANADA, OCTOBER 18-22, 1994. AMERICAN JOURNAL OF HUMAN GENETICS 55 (3 SUPPL.). 1994. A327. ISSN: 0002-9297, SHUTLER G G ET AL 'Characterization of cDNA clone that maps to the critical spinal muscular atrophy (SMA) locus region of 5q13.' ---</p>	1-34
A	<p>WO,A,92 00386 (UNIV COLUMBIA) 9 January 1992 see page 2, line 25 - line 32 see page 4, line 1 - page 8, line 22 see page 12, line 1 - page 13, line 2 see page 13, line 24 - line 30 see page 18, line 28 - page 21, line 6 see page 21, line 22 - page 23, line 28 ---</p>	1-34
P,X	<p>CELL (CAMBRIDGE, MASS.) (1995), 80(1), 167-78 CODEN: CELLB5; ISSN: 0092-8674, 1995 ROY, NATALIE ET AL 'The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy' see summary see page 169, right column, paragraph 2 - page 170, left column, paragraph 1 see page 171, right column, paragraph 3 - page 174, right column, paragraph 1 ---</p>	1-34
T	<p>NAT. MED. (N. Y.) (1995), 1(2), 124-7 CODEN: NAMEFI; ISSN: 1078-8956, 1995 GILLIAM, T. CONRAD 'Is the spinal muscular atrophy gene found' see page 125, middle column, paragraph 2 - right column, paragraph 2 see page 126, middle column, paragraph 2 - page 127, right column, paragraph 1 ---</p>	

-/--

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/CA 95/00581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>NAT. GENET. (1995), 9(2), 112-13 CODEN: NGENEC; ISSN: 1061-4036, 1995 MAHADEVAN, MANI S. ET AL 'SMA genes: deleted and duplicated' see the whole document -----</p>	

..information on patent family members

PCT/CA 95/00581

Form PCT/ISA/210 (patent family annex) (July 1992)

